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SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

Analogs of (±)-Epinephrine 437
F. F. Blicke and *W. K. Johnson*

α- and β-Amino Ketones and Corresponding Secondary Alcohols 440
F. F. Blicke and *W. K. Johnson*

1-Cycloalkyl-2-aminopropanes and Ethanes 443
F. F. Blicke and *W. K. Johnson*

Interference of Lactose in the Determination of Succinylsulfathiazole 446
José Luis Andrade, Juan Baumrucker, and *Víctor M. Márquez*

Bacitracin III. Formulatory Characteristics of Zinc Bacitracin 447
Herbert M. Gross, William A. Johnson, and *Grant J. Lafferty*

The Kinetics of the Hydrolysis of Atropine 450
Peter Zvirblis, Irving Socholitsky, and *Albert A. Kondritzer*

Assay of 2-Aminoheptane and Methyl Hexane Amine in Plastic Inhalers by Titration in Glacial Acetic Acid 454
J. P. Comer and *E. E. Kennedy*

A New Preparative Cell for High Voltage Electrophoresis 456
Rupert Salisbury and *E. P. Guth*

Amine Derivatives of Cyanuric Chloride II. Amino Esters 461
William O. Foye and *Lester Chafetz*

Chelating Agents as Color Stabilizers for Epinephrine Hydrochloride Solutions 464
Charles W. Roscoe and *Nathan A. Hall*

Prediction of Stability in Pharmaceutical Preparations III. Comparison of Vitamin Stabilities in Different Multivitamin Preparations 470
Edward R. Garrett

Evaluation of Collyria by the Rabbit Eye Method 474
James R. McCowan and *William J. Husa*

Synthesis of Isoniazid from Citric Acid 478
Manuel M. Baizer, Michael Dub, Sidney Gister, and *Nathan G. Steinberg*

Synthesis of Some Diacylimines 480
Donald E. Cadwallader, Jr., and *Joseph P. LaRocca*

The Physics of Tablet Compression XI. Determination of Porosity of Tablet Granulations 482
W. A. Strickland, Jr., L. W. Busse, and *T. Higuchi*

Evaluation of Substituted Amides Utilizing an Electronic Activity-Recording Device 487
Seldon D. Feurt and *Joseph P. LaRocca*

A Photometric Technique for the Quantitative Measurement of Changes in Peripheral Blood Flow 491
Laurence E. Gale and *Paul M. Scott*

A Study of the Anatomical Effects Produced in the Leaves of *Datura stramonium* L. by the Action of 2,4-Dichlorophenoxyacetic Acid 495
Charles Wassberg and *Forest J. Goodrich*

A Study of the Antibacterial Activity of Some Complex Aluminum Salts 498
Thelma N. Aguilar, Seymour M. Blaug, and *Louis C. Zopf*

Radioactive Iodine in Pharmaceutical Science 500
John E. Christian

A Note on the Melting Range of Succinylcholine Chloride 508
James E. Murphy and *C. W. Ferry*

A Note on the Synthesis of Pyrrole-2-carboxylic Acid 509
E. E. Smissman, M. B. Graber, and *R. J. Winzler*

Book Notices 509

Advertising ..... Cover pages 2, 3, 4, and I–VI

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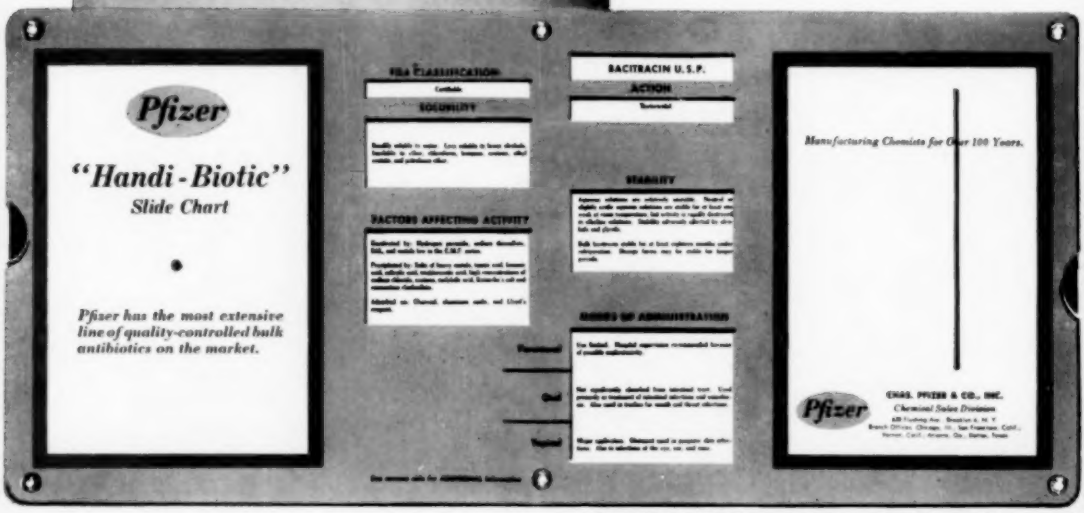

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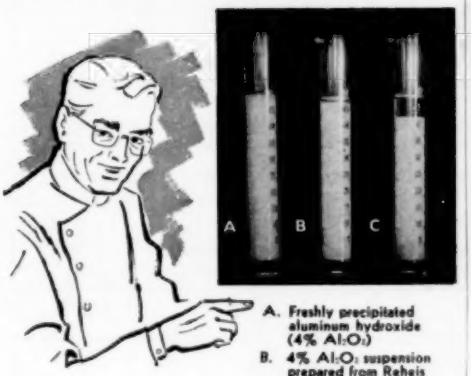

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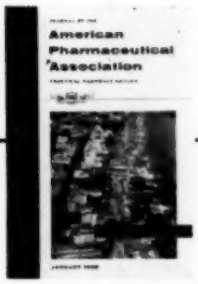

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(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ Occupying its customary position on the inside front cover, **Abbott Laboratories** takes occasion to delineate the efficacy of **Iberol,** indicated as particularly useful in treating the anemias.

▸ The inside back cover is this time given over to **Hoffmann-LaRoche,** whose timely insertion concerns **Tashan Cream,** a multi-vitamin cream for skin irritations.

▸ And, of course, the reader will find on the back cover a colorful insertion authored by **Hynson, Westcott & Dunning** publicizing its widely known **Mercurochrome.**

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VOLUME XLV — *JULY 1956* — NUMBER 7 — CONSECUTIVE No. 14

Analogs of (±)-Epinephrine*

By F. F. BLICKE and W. K. JOHNSON†

The interaction of (±)-epinephrine with hydrogen chloride and with hydrogen bromide in the presence of alcohols, and the treatment of (±)-epinephrine with thionyl chloride and then with alcohols have been studied. A cyclohexyl and a 3-cyclohexenyl analog of (±)-epinephrine were synthesized.

THE PREPARATION of (±)-epinephrine hydrochloride, by the use of alcoholic hydrogen chloride, has been described (1) but we found that the salt could be obtained pure and in a fairly satisfactory yield (77%) only when a specific amount of ethanol was employed. Two melting points have been reported for the salt: 157° (1) and "about 140°" (2). We found that the hydrochloride melted at 157° (decompn).

Treatment of (±)-epinephrine base with alcoholic hydrogen bromide did not yield the expected hydrobromide; instead, (±)-epinephrine ethyl ether hydrobromide (1-methylamino-2-ethoxy-2-(3,4-dihydroxyphenyl)-ethane hydrobromide) was produced. The corresponding methyl ether hydrobromide was obtained when methanol and hydrogen bromide were used.

Interaction of (±)-epinephrine with thionyl chloride yielded a viscous product which was crystallized from methanol-ether. This substance proved to be the hydrochloride of (±)-epinephrine methyl ether. It had been isolated previously by Funk and Freedman (3) after they had refluxed (±)-epinephrine hydrochloride with absolute methanol and hydrogen chloride, by Öppinger and Vetter (4) who used essentially the same method, and by Bretschneider (5) from the mother liquor after recrystallization of 1 - methylamino - 2 - chloro - 2 - (3,4 - diacetoxyphenyl)ethane hydrochloride from methanol-ethyl acetate.

Funk and Freedman (3) were also able to isolate the crystalline hydrochloride of (±)-epinephrine ethyl ether by the use of hydrogen chloride and absolute ethanol but they obtained only tarry material when propyl or benzyl alcohol was used in place of ethanol. By the substitution of hydrogen bromide for hydrogen chloride in the procedure of Funk and Freedman and the use of absolute ethanol, we obtained the hydrobromide of (±)-epinephrine ethyl ether. However, when propyl, isopropyl, butyl, benzyl, or β-phenylethyl alcohol or ethyl mercaptan was employed, only gummy material was obtained from the reaction mixtures. Although treatment of the viscous product, obtained from (±)-epinephrine and thionyl chloride, with absolute ethanol yielded the crystalline hydrochloride of (±)-epinephrine ethyl ether, the use of all of the alcohols mentioned above produced only intractable products.

In spite of the fact that catechol was converted into 1,2-cyclohexanediol in 95 per cent yield by low pressure hydrogenation in the presence of platinum oxide catalyst, neither (±)-epinephrine base nor the hydrochloride could be hydrogenated under the same conditions.

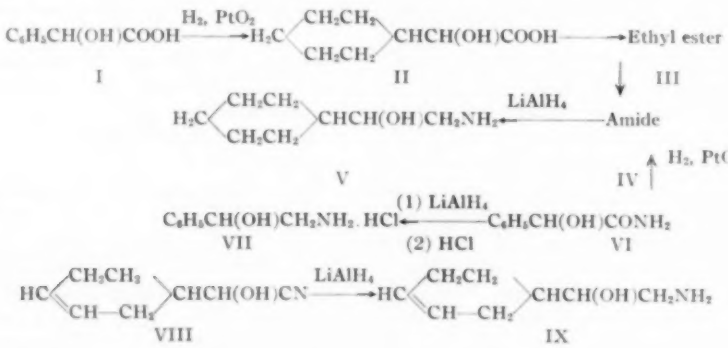
* Received March 31, 1956, from the College of Pharmacy, University of Michigan, Ann Arbor, Mich.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 13, 1956.
This paper represents part of a dissertation submitted by W. K. Johnson in partial fulfillment of the requirements for the Ph.D. degree in the University of Michigan, 1953.
† The Upjohn Company Fellow, 1950–1953.

It was found that the benzene ring was not attacked when O,O-diacetyladrenalone hydrochloride was hydrogenated with the aid of platinum oxide. The reaction product was the hydrochloride of O,O-diacetyl-(±)-epinephrine.

A cyclohexyl analog of epinephrine, 1-amino-2-hydroxy-2-cyclohexylethane (V) was obtained in the following manner. Hydrogenation of mandelic acid (I) with the aid of platinum oxide yielded hexahydromandelic acid (II) which was converted, successively, into the ethyl ester (III) (6) and the amide (IV) (7). Reduction of the amide with lithium aluminum hydride yielded the amine (V). The amide (IV) was also synthesized by hydrogenation of mandelamide (VI). Treatment of the amide (VI) with lithium aluminum hydride converted it into 1-amino-2-hydroxy-2-phenylethane (VII).

It was found that 1-amino-2-hydroxy-2-(3-cyclohexenyl)ethane (IX) can be obtained by reduction of the cyanohydrin of 3-cyclohexene-1-carboxaldehyde (VIII) with lithium aluminum hydride. Bromination of the hydrobromide of IX yielded 1-amino-2-hydroxy-2-(3,4-dibromophenyl)ethane hydrobromide.

The (±)-epinephrine analogs were tested in The Upjohn Company laboratories; none of them exhibited significant pressor activity.



EXPERIMENTAL

(±)-Epinephrine Hydrochloride.—(±)-Epinephrine base[1] (9.1 Gm.) was added to exactly 50 cc. of absolute ethanol which contained the amount of hydrogen chloride required for the formation of the hydrochloride. When the mixture was stirred, a brown solution was obtained and in about 10 minutes crystals began to separate. After 12 hours the copious precipitate was filtered and air-dried; yield 9.7 Gm.; m. p. 151–155° (decompn.). The hydrochloride was dissolved in the minimum amount of hot absolute ethanol (about 200 cc.) and after 4 days in a refrigerator the tan crystals were filtered; yield 8.5 Gm. (77%); m. p. 157° (decompn.).[2] Further recrystallization yielded colorless crystals, but the melting point did not change.

Anal.—Calcd. for $C_9H_{14}O_2NCl$: N, 6.37; Cl, 16.14. Found: N, 6.33; Cl, 16.04.

1 - Methylamino - 2 - methoxy - 2 - (3,4 - dihydroxyphenyl)ethane Hydrobromide.—(±)-Epinephrine base (1.8 Gm.) and 100 cc. of absolute methanol, which contained 2.5 Gm. of hydrogen bromide, were allowed to remain at room temperature for 48 hours. After 75 cc. of the solvent had been removed under reduced pressure, dry ether was added to the solution until it became turbid. The product, which precipitated gradually, weighed 2.2 Gm. (79%) after recrystallization from methanol-ether; m. p. 167–168° (decompn.).

Anal.—Calcd. for $C_{10}H_{16}O_3NBr$: N, 5.04; Br, 28.73. Found: N, 4.69; Br, 28.91.

1 - Methylamino - 2 - ethoxy - 2 - (3,4 - dihydroxyphenyl)ethane Hydrobromide.—(±)-Epinephrine base (1.8 Gm., 0.01 mole) was added to 10 cc. of absolute ethanol which contained 0.01 mole of hydrogen bromide. After 24 hours, dry ether was added until the solution became turbid. After some time the precipitate was filtered and recrystallized from ethanol-ether; yield 1.8 Gm. (64%); m. p. 176° (decompn.).

Anal.—Calcd. for $C_{11}H_{18}O_3NBr$: C, 45.24; H, 6.21; N, 4.80; Br, 27.37. Found: C, 45.54; H, 6.45; N, 4.79; Br, 27.20.

1 - Methylamino - 2 - methoxy - 2 - (3,4 - dihydroxyphenyl)ethane Hydrochloride.—(±)-Epinephrine hydrochloride (11.0 Gm.) was added to 100 cc. of freshly distilled thionyl chloride. The salt soon dissolved. After the solution had been refluxed for 30 minutes, the excess thionyl chloride was removed under reduced pressure. The viscous residue, which could not be crystallized, was mixed with 50 cc. of absolute methanol. After the heat of reaction had subsided, dry ether was added whereupon a crystalline, sulfur-free precipitate was obtained; m. p. 178–179° (decompn.) after recrystallization from methanol-ether; mixed m. p. with material ob-

[1] Purchased from Winthrop-Stearns.

[2] Reported melting points are 157° (1) and "about 140°" (2).

tained by the method of Funk and Freedman (3), 178–179° (decompn.); yield 10.0 Gm. (85.5%).

Anal.—Calcd. for $C_{10}H_{16}O_2NCl$: C, 51.39; H, 6.90; N, 5.99; Cl, 15.17. Found: C, 51.33; H, 6.35; N, 6.10; Cl, 15.43.

1 - Methylamino - 2 - ethoxy - 2 - (3,4 - dihydroxyphenyl)ethane Hydrochloride.—This compound was obtained in 50% yield in the manner described above with the use of absolute ethanol; m. p. and mixed m. p. 167–168° (decompn.).

Anal.—Calcd. for $C_{11}H_{18}O_3NCl$: N, 5.66; Cl, 14.32. Found: N, 5.45; Cl, 14.15.

1,2-Cyclohexanediol.—Catechol (11.0 Gm.) was dissolved in 50 cc. of 95% ethanol and 5 drops of 10% aqueous sodium hydroxide solution[3] and 0.3 Gm. of platinum oxide catalyst were added. Hydrogenation was carried out under an initial pressure of 50 pounds and was completed in 2 hours. After removal of the catalyst and the solvent, the residue was distilled; b. p. 128–130° (18 mm.); m. p. 82–84°; yield 11.0 Gm. (95%). The reported b. p. is 110–115° (5 mm.) (8) and the m. p. is 72–80° (8).

O,O-Diacetyl-(±)-epinephrine Hydrochloride.—O,O-Diacetyladrenalone hydrochloride (9) (8.7 Gm.), dissolved in 50 cc. of acetic acid, was hydrogenated under an initial pressure of 50 pounds in the presence of 0.3 Gm. of platinum oxide catalyst; yield 4.3 Gm. (49%) after recrystallization from acetone-ether; m. p. 164–165° (decompn.). The reported m. p. is 166° (10).

Hexahydromandelic Acid (II).—Mandelic acid (30.4 Gm.), dissolved in 150 cc. of acetic acid, was hydrogenated for 4 hours under an initial pressure of 50 pounds with the aid of 0.3 Gm. of platinum oxide catalyst. After removal of the catalyst and solvent, the residue was dissolved in the minimum amount of hot benzene. The cold solution yielded 29.5 Gm. (93%) of product; m. p. 133–134°. The reported m. p. is 134–135° (7).

Hexahydromandelamide (IV).—Mandelamide (11) (15.1 Gm.), dissolved in 100 cc. of acetic acid, was hydrogenated for 24 hours under an initial pressure of 50 pounds in the presence of 0.3 Gm. of platinum oxide catalyst. The residue obtained, after removal of the catalyst and solvent, was recrystallized from 250 cc. of boiling water; yield 10.2 Gm. (65%); m. p. 161–162°. The reported m. p. is 165.2° (corr.) (12).

1-Amino-2-hydroxy-2-cyclohexylethane (V).—The required amide 7.9 Gm., (0.05 mole) was added to a stirred mixture of 4.0 Gm. of lithium aluminum hydride and 200 cc. of anhydrous ether by the use of a modified Soxhlet extractor.[4] The mixture was refluxed for 12 hours and 8 cc. of water was then added, dropwise, to the cooled, stirred material. After filtration through a sintered glass filter, the precipitate was washed thoroughly with ether. The solvent was removed from the dried ethereal solution, and the residue was recrystallized from benzene; m. p. 133–135°; yield 4.0 Gm. (55%).

The hydrochloride, prepared by the use of ethereal hydrogen chloride, was recrystallized from isopropyl alcohol-ether; it softened at 205° and melted at 230–231°.

Anal.—Calcd. for $C_8H_{16}ONCl$: N, 7.79; Cl, 19.72. Found: N, 7.51; Cl, 19.76.

1-Amino-2-hydroxy-2-phenylethane Hydrochloride (VII).—Mandelamide (7.6 Gm., 0.05 mole) was allowed to react with 4.0 Gm. of lithium aluminum hydride in the manner described for (V). The dried ethereal solution was treated with hydrogen chloride whereupon 3.9 Gm. (45%) of the salt precipitated; m. p. 212° (decompn.). The reported m. p. is 211° (softened at 136°) (13).

1 - Amino - 2 - hydroxy - 2 - (3 - cyclohexenyl)-ethane.—A mixture of 300 cc. of water, 30.0 Gm. of sodium cyanide and 65.4 Gm. of 3-cyclohexene-1-carboxaldehyde[5] was stirred and cooled to 0°. A cooled solution of 33.2 Gm. of concentrated sulfuric acid in an equal volume of water was added, dropwise, at such a rate that the temperature of the mixture did not exceed 5°. The mixture was then stirred for 30 minutes, the oily layer was separated and the aqueous layer was extracted with ether. The combined oil and extract were dried with anhydrous magnesium sulfate. The ether solution was treated with 40.0 Gm. of lithium aluminum hydride in the usual manner; b. p. 135–140° (14 mm.); m. p. 39–40°; yield 50.0 Gm. (59%).

The hydrochloride, prepared by the use of ethereal hydrogen chloride, did not have a distinct melting point but softened at about 135°.

Anal.—Calcd. for $C_8H_{16}ONCl$: N, 7.93; Cl, 20.07. Found: N, 7.95; Cl, 20.26.

1 - Amino - 2 - hydroxy - 2 - (3,4 - dibromocyclohexyl)ethane Hydrobromide.—The hydrobromide of 1-amino-2-hydroxy-2-(3-cyclohexenyl)ethane[6] (8.8 Gm.), prepared by the use of ethereal hydrogen bromide, was suspended in 100 cc. of chloroform and 6.4 Gm. of bromine in 50 cc. of chloroform was added. The decolorization of the bromine was almost instantaneous. The solvent was removed under reduced pressure and the residue was recrystallized from isopropyl alcohol-ether; yield 13.6 Gm. (89%); m. p. 172–174° (decompn.) after softening at 165°.

Anal.—Calcd. for $C_8H_{16}ONBr_3$: C, 25.15; H, 4.22; Br^-, 20.93. Found: C, 25.12; H, 4.22; Br^-, 21.10.

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[3] See R. H. Levin, and J. H. Pendergrass, *J. Am. Chem. Soc.*, **69**, 2436(1947).

[4] See F. F. Blicke and Chi-Jung Lu, *J. Am. Chem. Soc.*, **74**, 3933(1952).

[5] Purchased from Distillation Products Industries, Division of Eastman Kodak Company.

[6] This compound softened at about 135° but did not have a characteristic melting point.

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α- and β-Amino Ketones and Corresponding Secondary Alcohols*

By F. F. BLICKE and W. K. JOHNSON†

Basic ketones of the types $RCOCH_2CH_2NR'_2$ and $RCOCH_2NR'_2$ were prepared by the use of the Mannich reaction or from a phenacyl halide. Some of the ketones were reduced to the corresponding secondary alcohols. A number of quaternary salts of the ketones and alcohols were synthesized.

BASIC KETONES of the types $RCOCH_2CH_2NR'_2$ (I) and $RCOCH_2NR'_2$ (II), as well as their corresponding secondary alcohols, have either been shown to exhibit pharmacological activity or to be useful for the synthesis of pharmacologically active products. The preparation of a number of such compounds as bases, hydrochlorides, and quaternary salts has been described in this paper. Compounds of type I were obtained by the use of the Mannich reaction; those of type II were prepared from phenacyl halides.

Mannich reactions were carried out by the use of acetovanillone, paraformaldehyde and the hydrochlorides of the following amines: dimethylamine, methylbenzylamine, hexamethylenimine, and heptamethylenimine. The products obtained are listed in Table I. The hydrochlorides of the Mannich bases prepared from dimethylamine and hexamethylenimine hydrochlorides were reduced with the aid of platinum oxide to the corresponding secondary alcohols.

We were unable to obtain Mannich bases when phenyl cyclohexyl ketone, isobutyrophenone or adrenalone hydrochloride was employed.[1]

Three basic ketones were synthesized by interaction of phenacyl chloride with hexamethylenimine and heptamethylenimine, respectively, and by reaction between chloroacetylcatechol and hexamethylenimine. Each ketone hydrochloride was reduced to the corresponding secondary alcohol with the use of platinum oxide.

Six quaternary salts of basic ketones (Table II) were obtained by interaction of phenacyl bromide, 3,4-dimethoxyphenacyl bromide or chloroacetylcatechol with tertiary amines such as trimethylamine, N-methylhexamethylenimine and N-methylheptamethylenimine. In two instances the quaternary salts of the basic ketones (compounds 3 and 5, Table II) were reduced to the corresponding secondary alcohols by the use of platinum oxide. In order to obtain methyl-N,N-heptamethylene - (2 - hydroxy - 2 - phenylethyl)ammonium bromide it was necessary to allow methyl bromide to react with 1-(1-heptamethylenimino)-2-hydroxy-2-phenylethane.

TABLE I.—HYDROCHLORIDES OF MANNICH BASES

$$\begin{matrix}(4)\ HO \\ (3)\ CH_3O\end{matrix}\!\!>C_6H_4COCH_2CH_2R.HCl$$

	R	M. p., °C.	Yield, %	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Chlorine Calcd.	Chlorine Found
1	Dimethylamino	184 (decompn.)	73	$C_{12}H_{18}O_3NCl$[a]	...	...	...	...	13.65	13.45
2	Methylbenzylamino	188–189 (decompn.)	90	$C_{18}H_{22}O_3NCl$	64.39	64.53	6.61	6.84	10.54	10.74
3	1-Hexamethylenimino[b]	166–168 (decompn.)[c]	60	$C_{14}H_{24}O_3NCl$	61.24	61.53	7.71	7.86	11.30	11.63
4	1-Heptamethylenimino	163–165 (decompn.)	46	$C_{17}H_{26}O_3NCl$	62.16	62.27	8.14	7.99	10.82	10.97

[a] Calcd. % N, 5.39; found, 5.40. [b] The methobromide melted at 164–165° (dec.) after recrystallization from methanol *Anal.*—Calcd. for $C_{17}H_{28}O_3NBr$: C, 54.85; H, 7.04; N, 3.76; Br, 21.47. Found: C, 54.65; H, 6.98; N, 3.83; Br, 21.40. [c] This product precipitated from the reaction mixture with one molecular equivalent of ethanol of crystallization which was lost when the substance was heated at 110° for 3 hours. Compounds 1, 2, and 4 were recrystallized from absolute ethanol.

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† The Upjohn Company Fellow, 1950–1953.

[1] See W. K. Johnson, Doctoral Dissertation, University of Michigan, 1953.

EXPERIMENTAL

Mannich Reactions.—Hydrochlorides of the following secondary amines were used in the general procedure described below: dimethylamine, methylbenzylamine (1), hexamethylenimine (2), and heptamethylenimine (2).

Acetovanillone[2] (0.1 mole), 4.0 Gm. of paraformaldehyde, 0.1 mole of the required amine hydrochloride and 200 cc. of absolute ethanol were refluxed for 24 hours. If the product did not separate from the cooled reaction mixture, the solvent was removed. The products are listed in Table I.

In the following two instances, the basic ketone salt was reduced to the corresponding secondary alcohol.

1 - (3 - Methoxy - 4 - hydroxyphenyl) - 3 - dimethylaminopropanol Hydrochloride.—The required basic ketone hydrochloride (10.0 Gm.) was dissolved in 50 cc. of acetic acid and hydrogenated in the presence of 0.3 Gm. of platinum oxide catalyst under an initial pressure of 45 pounds. The catalyst was removed by filtration and the solvent was removed under reduced pressure. The product melted at 171–172° (decompn.) after recrystallization from isopropyl alcohol; yield 4.0 Gm. (40%).

Anal.—Calcd. for $C_{12}H_{20}O_3NCl$: N, 5.35; Cl, 13.24. Found: N, 5.26; Cl, 13.36.

1 - (3 - Methoxy - 4 - hydroxyphenyl) - 3 - (1-hexamethylenimino)propanol Hydrochloride.—This compound was prepared in the manner described above in 87% yield; m. p. 148–150° (decompn.) after recrystallization from absolute ethanol.

Anal.—Calcd. for $C_{16}H_{26}O_3NCl$: C, 60.96; H, 8.25; N, 4.44; Cl, 11.23. Found: C, 61.03; H, 8.42; N, 4.55; Cl, 11.05.

Phenyl (1-hexamethylenimino)methyl Ketone Hydrochloride.—Phenacyl chloride (15.5 Gm., 0.1 mole), 9.9 Gm. (0.1 mole) of hexamethylenimine, 6.0 Gm. of anhydrous sodium carbonate and 100 cc. of absolute ethanol were refluxed for 4 hours. The cold mixture was filtered and ethereal hydrogen chloride was added to the filtrate to precipitate the product; yield 11.7 Gm. (46%); m. p. 222–223° (decompn.) after recrystallization from butanol.

Anal.—Calcd. for $C_{14}H_{20}ONCl$: N, 5.52; Cl, 13.97. Found: N, 5.16; Cl, 14.14.

Phenyl (1-heptamethylenimino)methyl Ketone Hydrochloride.—This product was obtained in the manner described above by the use of heptamethylenimine; m. p. 220–221° (decompn.) after recrystallization from butanol; yield 43%.

Anal.—Calcd. for $C_{15}H_{22}ONCl$: N, 5.23; Cl, 13.24. Found: N, 5.10; Cl, 13.33.

3,4-Dihydroxyphenyl (1-hexamethylenimino)-methyl Ketone Hydrochloride.—This substance was prepared in the described manner with the use of chloroacetylcatechol[2] and hexamethylenimine except that the reaction mixture was not heated but was allowed to remain at room temperature for 24 hours; m. p. 241–242° (decompn.) after recrystallization from methanol-ether; yield 47%.

Anal.—Calcd. for $C_{14}H_{20}O_3NCl$: C, 58.75; H, 7.06; N, 4.90; Cl, 12.41. Found: C, 58.95; H, 7.30; N, 4.87; Cl, 12.54.

1 - (1 - Hexamethylenimino) - 2 - hydroxy - 2-phenylethane Hydrochloride.—The required basic ketone hydrochloride (10.2 Gm., 0.04 mole) was dissolved in 100 cc. of water and hydrogenated in the presence of 0.3 Gm. of platinum oxide catalyst under an initial pressure of 50 pounds; m. p. 173–174° (decompn.) after recrystallization from ethanol-ether; yield 9.1 Gm. (88%).

Anal.—Calcd. for $C_{14}H_{22}ONCl$: N, 5.48; Cl, 13.86. Found: N, 5.38; Cl, 13.96.

1 - (1 - Hexamethylenimino) - 2 - hydroxy - 2-(3,4-dihydroxyphenyl)ethane Hydrochloride.—The required basic ketone hydrochloride was reduced in the described manner; m. p. 161–163° (decompn.) after recrystallization from butanol; yield 86%.

Anal.—Calcd. for $C_{14}H_{22}O_3NCl$: C, 58.42; H, 7.71; N, 4.87; Cl, 12.32. Found: C, 58.72; H, 7.85; N, 4.22; Cl, 12.36.

1 - (1 - Heptamethylenimino) - 2 - hydroxy - 2-phenylethane Hydrochloride.—By the described process, this compound was obtained in 65% yield from the required ketone salt; m. p. 152–153° (decompn.)after recrystallization from isopropyl alcohol.

Anal.—Calcd. for $C_{15}H_{24}ONCl$: N, 5.19; Cl, 13.14. Found: N, 5.13; Cl, 13.21.

N - Methylhexamethylenimine.—Hexamethylenimine (2) (29.7 Gm., 0.3 mole), 46.5 Gm. of 35% formaldehyde, and 76.5 Gm. of 90% formic acid were heated on a steam bath for 12 hours. After the addition of 35 cc. of concentrated hydrochloric acid to the cold mixture, the solution was evaporated to dryness on a steam bath. The residue was dissolved in a minimum amount of water, sodium hyoxide was added to the solution and the amine was extracted with ether. From the dried extract, 27.7 Gm. (81.5%) of product was obtained; b. p. 137–139° (740 mm.).[4]

For characterization, the base was converted into the hydrochloride; m. p. 182–183° (decompn.).

N-Methylheptamethylenimine.—By the method described above, 113.0 Gm. of heptamethylenimine (2) yielded 97.3 Gm. (76.5%) of product; b. p. 159–161° (734 mm.). The hydrochloride melted at 162–163° (decompn.). The reported m. p. is 163–164° (2).

3,4-Dimethoxyacetophenone.—A mixture of 166 Gm. of acetovanillone and 450 cc. of water was stirred and heated on a steam bath. A solution was prepared from 150 Gm. of sodium hydroxide and 750 cc. of water, and 360 cc. of this solution was added to the mixture. Methyl sulfate (142 cc.) was then added at such a rate that the mixture refluxed. The mixture was refluxed for 45 minutes. An additional 30 cc. of methyl sulfate was added, followed shortly by 60 cc. of sodium hydroxide solution. After the last mentioned operation had been repeated two more times, the mixture was made strongly alkaline, extracted with ether, and then the extract was dried with magnesium sulfate. The product boiled at 158–160° (8 mm.); m. p. 52–53°; yield 155 Gm. (86%). The reported b. p. is 158° (9 mm.) (3) and the m. p. is 51° (corr.) (4).

The quaternary salts of the basic ketones (Table II) were obtained in the following manner.

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[2] Obtained from the Marathon Corporation, Chemical Division, Rothschild, Wis.

[3] Purchased from Winthrop-Stearns.

[4] G. R. Toy, (Doctoral Dissertation, University of Michigan, 1951) prepared the base by a different method; b. p. 139–140°; m. p. of the hydrochloride, 181.5–183.5°.

Compounds 1 and 2.—Phenacyl bromide (0.1 mole), 0.1 mole of N-methylhexamethylenimine (or N-methylheptamethylenimine) and 150 cc. of absolute ethanol were refluxed for 4 hours, and ether was then added to the cold mixture to precipitate the product.

Compound 3.—Chloroacetylcatechol (10.8 Gm.), 4.8 Gm. of trimethylamine and 100 cc. of absolute ethanol were allowed to react at room temperature for 24 hours whereupon the product precipitated.

Compound 4.—Chloroacetylcatechol (5.6 Gm.), 3.4 Gm. of N-methylhexamethylenimine and 50 cc. of absolute ethanol were allowed to react at room temperature for 24 hours. The solvent was removed, the residue was dissolved in 2 liters of boiling water, and 10 Gm. of picric acid was added. The picrate precipitated from the cold solution.

Compounds 5 and 6.—A mixture of 10.4 Gm. of 3,4-dimethoxyphenacyl bromide, 4.5 Gm. of N-methylhexamethylenimine (or N-methylheptamethylenimine) and 50 cc. of absolute ethanol was refluxed for 4 hours. Ether was added to the cold mixture to precipitate the product.

Trimethyl - [2 - hydroxy - 2 - (3,4 - dihydroxyphenyl)ethyl]ammonium Chloride.—Trimethyl-3,4-dihydroxyphenacylammonium chloride (compound 3, Table II) (12.6 Gm., 0.06 mole), dissolved in 50 cc. of water, was hydrogenated under an initial pressure of 50 pounds in the presence of 0.3 Gm. of platinum oxide catalyst. The catalyst was removed by filtration and the solvent was removed under reduced pressure; m. p. 172–173° (decompn.) after recrystallization from methanol-ether; yield 5.0 Gm. (40%).

Anal.—Calcd. for $C_{11}H_{16}O_3NCl$: N, 5.66; Cl, 14.32. Found: N, 5.60; Cl, 14.19.

Methyl - N,N - hexamethylene - (2 - hydroxy - 2-phenylethyl)ammonium Bromide.—The quaternary bromide (compound 5, Table II) was hydrogenated in the manner described above; m. p. 173–174° (decompn.); yield 66%.

Anal.—Calcd. for $C_{15}H_{24}ONBr$: C, 57.31; H, 7.92; N, 4.46; Br, 25.42. Found: C, 57.44; H, 7.70; N, 4.19; Br, 25.64.

TABLE II.—QUATERNARY SALTS OF BASIC KETONES

$$RCOCH_2\overset{+}{R'}\,X^-$$

	R	R′	X⁻	M. p., °C.	Yield, %
1	C_6H_5	$N(CH_2)_6(CH_3)$	Br	162–163 (decompn.)	64
2	C_6H_5	$N(CH_2)_7(CH_3)$	Br	183–185 (decompn.)	91
3	$3,4\text{-}(HO)_2C_6H_3$	$N(CH_3)_3$	Cl	234–235 (decompn.)	85
4	$3,4\text{-}(HO)_2C_6H_3$	$N(CH_2)_6(CH_3)$	Picrate	169–171 (decompn.)	86
5	$3,4\text{-}(CH_3O)_2C_6H_3$	$N(CH_2)_6(CH_3)$	Br	203–204 (decompn.)	73
6	$3,4\text{-}(CH_3O)_2C_6H_3$	$N(CH_2)_7(CH_3)$	Br	167–168 (decompn.)	64

		Analyses, %							
		Carbon		Hydrogen		Nitrogen		Halogen	
	Formula	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found
1	$C_{15}H_{22}ONBr$	57.68	57.90	7.10	7.41	4.49	4.45	25.60	25.65
2	$C_{16}H_{24}ONBr$	58.90	58.73	7.41	7.66	4.29	4.07	24.50	24.40
3	$C_{11}H_{16}O_3NCl$	...	...	...	...	5.70	5.66	14.43	14.37
4	$C_{21}H_{24}O_{10}N_4$	...	...	...	...	11.38	11.56	...	...
5	$C_{17}H_{26}O_3NBr$	54.85	54.82	7.04	7.12	3.76	3.69	21.47	21.38
6	$C_{18}H_{28}O_3NBr$	55.97	55.63	7.32	7.52	3.63	3.37	20.69	20.53

Compound 1 was recrystallized from ethanol-ether; 3 from methanol; 5 from ethanol; 6 from isopropyl alcohol.

Methyl - N,N - heptamethylene - (2 - hydroxy - 2-phenylethyl)ammonium Bromide.—When the quaternary bromide (compound 6, Table II) was hydrogenated as described above, a mixture of products was obtained from which methylphenylcarbinol was isolated in 55% yield. The carbinol was identified as the α-naphthylurethane; m. p. and mixed m. p. 105°. The reported m. p. is 106° (5).

The desired compound was prepared by the addition of a slight excess of methyl bromide to an ethereal solution of 1-(1-heptamethylenimino)-2-hydroxy-2-phenylethane. After a week the salt had precipitated in 45% yield; m. p. 167–169° (decompn.) after recrystallization from butanol-ether.

Anal.—Calcd. for $C_{16}H_{26}ONBr$: C, 58.53; H, 7.98; N, 4.27; Br, 24.35. Found: C, 58.27; H, 8.04; N, 4.23; Br, 24.18.

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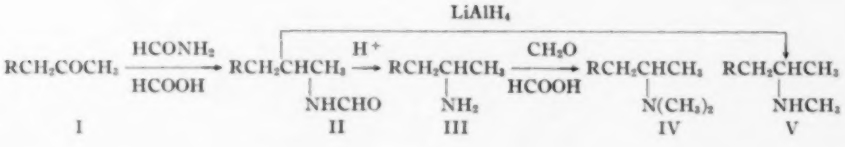
1-Cycloalkyl-2-aminopropanes and Ethanes*

By F. F. BLICKE and W. K. JOHNSON†

A number of cycloalkylacetones were prepared by treatment of a cycloalkylacetyl chloride with dimethylcadmium. The acetones were converted into formamido derivatives from which, by simple operations, primary, secondary and tertiary aminopropanes were obtained. From 1-cycloheptyl-2-aminoethane the corresponding N-methyl and N,N-dimethyl derivatives were synthesized.

A NUMBER of 1-cycloalkyl-2-aminopropanes were prepared as bases and were converted into their hydrohalide and methobromide salts.

In order to obtain the bases, the substituted acetones (I) were allowed to react with formamide and formic acid, and the formamido derivatives (II) obtained were hydrolyzed to the primary amines (III). Treatment of the primary amines with formaldehyde and formic acid converted them into the tertiary amines (IV), and reduction of the formamido derivatives with lithium aluminum hydride produced the secondary amines (V).



R = Cyclohexyl, cycloheptyl, 4-methylcycloheptyl, or cyclooctyl.

The substituted acetones were synthesized by preparation of the required cycloalkyl bromide, conversion of the bromide into the corresponding acetic acids by the malonic ester synthesis,[1] preparation of the acetyl chloride, and interaction of the acid chloride with dimethylcadmium.[2]

1-Cycloheptyl-2-methylaminoethane was obtained by treatment of 1-cycloheptyl-2-aminoethane with chloral[3] and reduction of the N-formyl derivative with lithium aluminum hydride.

1-Cycloheptyl-2-dimethylaminoethane was prepared by methylation of the corresponding primary amine with formaldehyde and formic acid.

EXPERIMENTAL

All of the required cyclohexyl intermediates are known compounds, and cycloheptylacetyl chloride (1) has been described recently.

4-Methylcycloheptyl Bromide.—The necessary 4-methyl-1-aminomethylcyclohexanol hydrochloride, prepared from 4-methylcyclohexanone cyanohydrin (2, 3), which had not been distilled, was recrystallized twice from 95% ethanol and then from isopropyl alcohol; m. p. 210–211° (decompn.). The reported melting points are 165° (2b) and 194–195° (4).

Anal.—Calcd. for $C_8H_{18}ONCl$: N, 7.80; Cl, 19.72. Found: N, 7.75; Cl, 19.87.

The cyclohexanol hydrochloride was converted into 4-methylcycloheptanone (2) which was reduced to 4-methylcycloheptanone in 75% yield with lithium aluminum hydride; b. p. 108–112° (40 mm.). The reported b. p. is 105–106° (39–40 mm.) (3).

A solution of 136.0 Gm. of phosphorus tribromide in an equal volume of dry benzene was added, dropwise, to a stirred solution of 95.3 Gm. of 4-methylcycloheptanol in 450 cc. of benzene. The mixture was heated on a steam bath for 1 hour, the benzene was removed, the residue was poured into ice-water and the oily layer was separated. The aqueous layer was extracted with ether, and the combined extract and oil were dried. The 4-methylcycloheptyl bromide boiled at 90–95° (20 mm.); yield 114.0 Gm. (79.5%).

Anal.—Calcd. for $C_8H_{15}Br$: Br, 41.83. Found: Br, 41.79.

Cyclooctyl Bromide.—Cyclooctatetraene[4] (120.0 Gm.) was reduced to cyclooctene (5). The crude product was hydrobrominated in acetic acid (6). The yield of bromide was 178.0 Gm. (93.5%); b. p. 104–108° (18 mm.). The reported b. p. is 90.5–91.5° (10 mm.) (6).

4-Methylcycloheptylmalonic Acid.—Diethyl 4-methylcycloheptylmalonate was prepared in the usual manner from 2.3 Gm. of sodium, 100 cc. of

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† The Upjohn Company Fellow, 1950–1953.

[1] In some instances, the malonic ester and acid were not isolated.

[2] The general procedure described by J. Cason and F. S. Prout (*Organic Syntheses*, **28**, 75(1948)) was employed in this last reaction.

[3] For formylation by the use of chloral see F. F. Blicke and Chi-Jung Lu, *J. Am. Chem. Soc.*, **74**, 3933(1952).

[4] Purchased from General Aniline and Film Corporation.

TABLE I.—SUBSTITUTED ACETONES

RCH_2COCH_3					Semicarbazones				
						Analyses, %			
						Carbon		Hydrogen	
R	B. p., °C.	Mm.	Yield, %	M. p., °C. (decompn.)	Formula	Calcd.	Found	Calcd.	Found
A[a]	93–96[b]	26	68	173–174[b]	. . .	. . .	. . .	. . .	. . .
B	110–113[c]	18	58	178–179	$C_{11}H_{21}ON_3$	62.81	62.44	9.59	9.54
C	108–112	10	76	162–163	$C_{12}H_{23}ON_3$	63.96	63.92	10.29	10.34
D	122–128	14	75	183–184	$C_{12}H_{23}ON_3$	63.96	64.14	10.29	10.28

[a] A = Cyclohexyl, B = cycloheptyl, C = 4-methylcycloheptyl, D = cyclooctyl.
[b] O. Wallach, (*Ann.*, **394**, 377(1912)), b. p. 200°, m. p. 171–172°.
[c] W. C. McCarthy and T. H. Brown (THIS JOURNAL, **43**, 661(1954)), b. p. 98–99° (14 mm.). All semicarbazones were recrystallized from methanol.

absolute ethanol, 19.2 Gm. of diethyl malonate and 18.2 Gm. of methylcycloheptyl bromide. The ester boiled at 163–167° (10 mm.); yield 16.5 Gm. (63%).

A mixture of 6.0 Gm. of potassium hydroxide, 6 cc. of water and 10.8 Gm. of the ester was heated for 4 hours on a steam bath. After the addition of 15 cc. of concentrated hydrochloric acid to the ice-cold mixture, the precipitate was recrystallized from toluene; yield 5.2 Gm.; m. p. 113–114°.

Anal.—Calcd. for $C_{11}H_{18}O_4$: C, 61.64; H, 8.46; neut. equiv., 214.2. Found: C, 61.87; H, 8.53; neut. equiv., 212.4.

4-Methylcycloheptylacetic Acid.—To an alcoholic solution of sodium ethylate, prepared from 11.5 Gm. of sodium and 500 cc. of absolute ethanol, there was added 90.0 Gm. of diethylmalonate, and the mixture was stirred for 2 hours. 4-Methylcycloheptyl bromide (95.5 Gm.) was added and the mixture was refluxed for 3 days. The sodium bromide was filtered from the cold mixture and the alcohol was removed by distillation. A solution of 84.0 Gm. of potassium hydroxide in 100 cc. of water was added to the residue, and the mixture was heated on a steam bath for 3 hours. After cooling, 150 cc. of concentrated hydrochloric acid was added, dropwise, with stirring. The oily layer was separated and the aqueous portion was extracted with ether. The combined oil and extract were dried with anhydrous magnesium sulfate. The ether was removed and the residue was heated with 1.0 Gm. of copper-bronze under reduced pressure (water pump). When the decarboxylation was completed, the material was distilled; b. p. 105–107° (0.7 mm.).

Anal.—Calcd. for $C_{10}H_{18}O_2$: C, 70.54; H, 10.66. Found: C, 70.50; H, 10.56.

Cyclooctylacetic Acid.—From 191.0 Gm. of cyclooctyl bromide there was obtained, without isolation of the malonic ester or the malonic acid, in the manner described above, 65.0 Gm. (42.5%) of the acetic acid; b. p. 125–129° (0.5 mm.). The reported b. p. is 130° (1 mm.) (7).

The alcohol, used in the preparation of the malonic ester, was diluted with 4 liters of water. The immiscible oily layer was separated and the aqueous layer was extracted with ether. From the combined oil and extract, 21.0 Gm. of cyclooctene was obtained; b. p. 62–64° (50 mm.).

4-Methylcycloheptylacetyl Chloride and 4-Methylcycloheptylacetamide.—To 47.6 Gm. (0.4 mole) of thionyl chloride, cooled in an ice bath, there was added, dropwise, 51.0 Gm. (0.3 mole) of 4-methylcycloheptylacetic acid. The mixture was refluxed for 30 minutes and then fractionated; the acid chloride boiled at 114–117° (14 mm.); yield 87%.

The amide, obtained by adding the acid chloride to cold concentrated ammonia water, was purified by dissolving it in chloroform and cooling the solution in a dry ice-acetone bath. The precipitated amide melted at 128–130°.

Anal.—Calcd. for $C_{10}H_{19}ON$: C, 70.93; H, 11.31; N, 8.25. Found: C, 70.94; H, 11.45; N, 7.96.

Cyclooctylacetyl Chloride and Cyclooctylacetamide.—Prepared in the manner described above, the acid chloride boiled at 128–132° (18 mm.); yield 88%.

The amide, after recrystallization from chloroform, melted at 130–132°.

Anal.—Calcd. for $C_{10}H_{19}ON$: C, 70.93; H, 11.31; N, 8.25. Found: C, 70.35; H, 11.26; N, 7.75.

1-Cycloalkyl-2-formamidopropanes.—A mixture of 0.12 mole of the required ketone, 45.0 Gm. of formamide and 20.0 Gm. of 90% formic acid was refluxed (bath temperature 160–180°) for 6 hours with continuous removal of water. The cold mixture was extracted with ether and the extract was dried with magnesium sulfate.

The 1-cyclohexyl compound boiled at 177–180° (18 mm.); yield 61%.

Anal.—Calcd. for $C_{10}H_{19}ON$: N, 8.27. Found: N, 8.22.

The 1-cycloheptyl compound boiled at 187–191° (14 mm.); yield 54%.

The boiling point of the 1-(4-methylcycloheptyl) compound was 179–184° (8 mm.) (yield 55%) and that of the 1-cyclooctyl compound was 195–200° (13 mm.) (yield 55%).

Examples which illustrate the general procedures for the synthesis of the primary, secondary, and tertiary amines are reported below.

1-(4-Methylcycloheptyl)-2-aminopropane.—A mixture of 7.3 Gm. of the required formylaminopropane, 50 cc. of concentrated hydrochloric acid, and 20 cc. of water was refluxed for 2 hours. After 32 Gm. of sodium hydroxide, dissolved in 100 cc. of water, had been added to the cold mixture, it was extracted with ether. The product was obtained by distillation.

1-(4-Methylcycloheptyl)-2-methylaminopropane.—The required formylaminopropane (5.9 Gm.) was allowed to react with 2.0 Gm. of lithium aluminum hydride in the usual manner (8). The amine was purified by distillation.

1-(4-Methylcycloheptyl)-2-dimethylaminopropane.—A mixture of 3.5 Gm. of 1-(4-methylcycloheptyl)-2-aminopropane, 7.0 Gm. of 35% aqueous formaldehyde, and 7.0 Gm. of formic acid was heated on a steam bath for 24 hours. Concentrated hydrochloric acid (10 cc.) was added to the cold mixture and it was evaporated to dryness on a steam bath.

TABLE II.—1-CYCLOALKYL-2-AMINOPROPANES, BASES, AND SALTS

$RCH_2CH(CH_3)NR'R''$									Analyses, %			
			B. p.,		Yield,		M. p.,		Nitrogen		Halogen	
R	R'	R''	°C.	Mm.	%	Salt	°C.	Formula	Calcd.	Found	Calcd.	Found
A[a]	H	H	82–85[b]	18	68	HCl	190–191[b]	...	...	...	...	...
A	H	CH_3	107–109[c]	40	92	HCl	127–129[c]	$C_{10}H_{22}NCl$	7.31	7.38	18.49	18.51
B	H	H	97–98[d]	12	72	HCl	209[d]	$C_{10}H_{22}NCl$	7.31	7.47	18.49	18.42
B	H	CH_3	129–131[e]	40	82	HCl	135–137[e]	$C_{11}H_{24}NCl$	6.81	6.97	17.23	17.54
B	CH_3	CH_3	122–123	20	76	HCl	153–154	$C_{12}H_{26}NCl$	6.38	6.42	16.13	16.30
B	CH_3	CH_3	...	..	..	CH_3Br	272–273	$C_{13}H_{28}NBr$	5.02	5.16	28.72	28.91
C	H	H	99–101	9	68	HCl	178–180	$C_{11}H_{24}NCl$	6.81	7.02	17.23	17.42
C	H	CH_3	105–107	8	78	HCl	102–103	$C_{12}H_{26}NCl$	6.38	6.43	16.13	16.06
C	CH_3	CH_3	109–111	10	82	HBr	128–129	$C_{13}H_{28}NBr$	5.02	4.99	28.72	28.74
C	CH_3	CH_3	...	..	..	CH_3Br	247–251	$C_{14}H_{30}NBr$[f]	4.79	4.51	27.35	27.33
D	H	H	112–115	11	75	HCl	182–184	$C_{11}H_{24}NCl$	6.81	6.69	17.23	17.09
D	H	CH_3	124–126	14	89	HCl	129–131	$C_{12}H_{26}NCl$	6.38	6.39	16.13	16.14
D	CH_3	CH_3	137–140	21	80	HBr	140–141	$C_{13}H_{28}NBr$	5.02	5.18	28.72	28.93
D	CH_3	CH_3	...	..	..	CH_3Br	248–250	$C_{14}H_{30}NBr$[g]	4.79	4.66	27.35	27.30

[a] A = Cyclohexyl, B = cycloheptyl, C = 4-methylcycloheptyl, D = cyclooctyl. [b] B. L. Zenitz, E. B. Macks, and M. L. Moore (*J. Am. Chem. Soc.*, **69**, 1117(1947)), b. p. 85–87° (21 mm.), m. p. 191–192°. [c] Ref. (*b*), b. p. 92–93° (20 mm.), m. p. 127–128°. [d] W. C. McCarthy and T. H. Brown (THIS JOURNAL, **43**, 661(1954)), b. p. 101–102° (18 mm.), m. p. 210–211°. [e] Ref. (*d*), b. p. 111–112° (19 mm.), m. p. 135–136°. [f] Calcd.: C, 57.52; H, 10.35. Found: C, 57.37; H, 10.22. [g] Calcd.: C, 57.52; H, 10.35. Found: C, 58.03; H, 10.34. The methobromides were recrystallized from isopropyl alcohol-ether.

The residue was dissolved in a few cubic centimeters of water, and concentrated sodium hydroxide solution was added. After separation of the oil and extraction of the aqueous layer with ether, the combined oil and extract were dried and distilled.

1-Cycloheptyl-2-formylaminoethane.—From 1-cycloheptyl-2-aminoethane[5] (14.0 Gm.) and 14.7 Gm. of chloral, 14.7 Gm. (87%) of product was obtained; b. p. 140–145° (0.7 mm.).

Anal.—Calcd. for $C_{10}H_{19}ON$: N, 8.27. Found: N, 8.14.

1-Cycloheptyl-2-methylaminoethane.—Reduction of 14.7 Gm. of the formyl compound with 4.0 Gm. of lithium aluminum hydride yielded 8.7 Gm. (64.5%) of the amine; b. p. 108–109° (20 mm.). The hydrochloride melted at 182–183° (decompn.).

Anal.—Calcd. for $C_{10}H_{22}NCl$: N, 7.31; Cl, 18.49. Found: N, 7.20; Cl, 18.69.

[5] The preparation of this amine has been described by Juan Azuara S. (Doctoral Dissertation, University of Michigan, 1954). It was obtained by reduction of β-cycloheptenylacetonitrile with lithium aluminum hydride and then hydrogenated to the 1-cycloheptenyl-2-aminoethane with platinum oxide catalyst. The nitrile and the saturated amine have been synthesized by W. C. McCarthy and T. H. Brown (1).

1-Cycloheptyl-2-dimethylaminoethane.—A mixture of 28.2 Gm. of the primary amine, 45.0 Gm. of 89–90% formic acid and 40.0 Gm. of 35% aqueous formaldehyde was treated in the manner described above; b. p. 117–121° (28 mm.); yield 27.0 Gm. (80%). The hydrochloride melted at 132–133° (decompn.).

Anal.—Calcd. for $C_{11}H_{24}NCl$: N, 6.65; Cl, 17.23. Found: N, 6.84; Cl, 17.21.

The methobromide melted at 242° (decompn.) after recrystallization from isopropyl alcohol-ether.

Anal.—Calcd. for $C_{12}H_{26}NBr$: N, 5.30; Br, 30.24. Found: N, 5.29; Br, 30.12.

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Papers for 1956 Meeting of the A. A. A. S.

The 1956 meeting of the American Association for the Advancement of Science will be held in New York City the week starting December 26. The tentative program of the Pharmacy Section Meetings includes the following features: (1) Contributed papers, December 26, 8:00 p. m., and December 27, 9:00 a. m.; (2) Three symposiums on "Cosmetics in Medical Practice," "Compressed Coating Techniques and Equipment," and "Anti-enzymes," December 27, 28, and 29, respectively; (3) Hospital Pharmacy—Contributed Papers, December 29, 9:00 a. m. For further information see *Science* or *Scientific Monthly* beginning in the May and June issues.

A paper should require not more than fifteen minutes for presentation. Authors who wish to present papers should send titles and two copies of a short abstract as soon as possible, but not later than September 21, 1956, to Dr. John E. Christian, *Secretary,* Purdue University School of Pharmacy, Lafayette, Ind.

Interference of Lactose in the Determination of Succinylsulfathiazole*

By JOSÉ LUIS ANDRADE, JUAN BAUMRUCKER, and VICTOR M. MARQUEZ

When trying to evaluate succinylsulfathiazole in the presence of lactose by the method described in U. S. P. XV, pages 680–682, an interference was observed which resulted in a higher consumption of the nitrite solution. If the determination is performed after acid hydrolysis, exact results are obtained and the method is of more general application.

THE METHOD DESCRIBED in the U. S. P. XV, pages 680–682, for the assay of succinylsulfathiazole is well known and gives satisfactory results in the assay of the pure drug. When this method is applied in presence of lactose, an interference is introduced which produces abnormally high and inconsistent results. This interference was observed during the control of the succinylsulfathiazole content in a pharmaceutical specialty[1] which also contained lactose among other ingredients.

EXPERIMENTAL

A series of tests were performed to determine the amount of succinylsulfathiazole in different fractions of the mixture, because when working on the entire product, abnormally high and inconsistent results were obtained: (*a*) On the alkalinized aqueous fraction (using sodium bicarbonate or sodium hydroxide) to make the succinylsulfathiazole soluble and separate it from other insoluble ingredients (bentonite, tricalcium phosphate); (*b*) On the residue resulting from the exhaustion of the substances soluble in water at room temperature (24°) (separation from lactose, dihydrostreptomycin, sodium citrate, B-complex vitamin); and (*c*) On the acetone extract of the dry residue containing the free sulfathiazole obtained by alkaline hydrolysis and subsequent acidification.

As can be observed from Table I, the high results obtained in Fraction 1 reveal that the interfering ingredient is present among the soluble substances in the alkalinized water. In Fraction 2, the results are very low, which proves that the interfering substance has been eliminated by the water. On the other hand, in spite of its slight solubility, some of the succinylsulfathiazole was dissolved in the volume of the solvent employed. The results obtained on the Fraction 3 are not accurate probably because of the multiplicity of operations performed in addition to the difficulty of extracting the residuum of free sulfathiazole completely.

TABLE I

	SS[a] in 5 Gm. of the Mixture, mg.	0.1 *M* $NaNO_2$ Equiv. to the SS, ml.	0.1 *M* $NaNO_2$ Consumed, ml.	SS Equiv. to ml. 0.1 *M* $NaNO_2$ Consumed, mg.
Fraction 1[b]	200	5.36	14.0	522.76
Fraction 2	200	5.36	2.94	109.78
Fraction 3	200	5.36	4.60	171.76

[a] SS = succinylsulfathiazole.
[b] Average of 3 determinations in each case.

In order to determine, with precision, which of the substances soluble in water was causing the interference, new tests were subsequently made on prepared samples. Each specimen contained succinylsulfathiazole and one of the soluble ingredients. This procedure made it evident that lactose was the cause of interference and could be proved definitely when samples of pure lactose were subjected to the same procedure of determination as the succinylsulfathiazole already named. This included a prior hydrolysis in an alkaline medium and consumed large, but relatively regular and constant quantities of nitrite solution (see Table II). On the other hand, when applying the method to pure lactose without alkaline hydrolysis or with acid hydrolysis, no abnormal consumption of nitrite solution results.

TABLE II[a]

Lactose Present, Gm.	0.1 *M* $NaNO_2$ Consumed, ml.
0.10	0.1
0.40	0.7
1.00	1.7
2.00	3.4
3.00	6.5
4.00	9.2

[a] Figures shown on this Table are ml. of $NaNO_2$ 0.1 *M* required for pure lactose after alkaline hydrolysis in amount of sugar from 0.1 Gm. up to 4 Gm. As noted from same the number of milliliters of $NaNO_2$ are relatively constant and are in some relation with the different amounts of sugar present.

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[1] Streptosuccinil Cosmos (Powder)—Each dose (5 Gm.) contains: dihydrostreptomycin sulfate 0.0525 Gm., succinylsulfathiazole 0.200 Gm., apple pectin 0.075 Gm., dried aluminum hydroxide 0.240 Gm., sodium citrate 0.100 Gm., excipient q.s. The excipient is composed of lactose 4.32 Gm., bentonite, tricalcium phosphate, thiamine hydrochloride 2 mg., riboflavin 1 mg., niacinamide 10 mg. and folic acid 0.1 mg.

METHOD

Considering the inconvenience presented by the alkaline hydrolysis of succinylsulfathiazole in presence of lactose, which does not occur in acid medium we verified the determination with the diazotization procedure, but used a prior hydrolysis in an acid medium, as follows:

Weigh exactly a sufficient quantity of the product equivalent to 500 mg. of succinylsulfathiazole. Add 80 ml. of hydrochloric acid (1 + 2) and boil at reflux for an hour. Cool, add ice, and titrate with a 0.1 *M* sodium nitrite solution, employing as indicator potassium iodide-starch paper according to the usual technique. Each ml. of 0.1 *M* sodium nitrite is equivalent to 0.03734 Gm. of succinylsulfathiazole monohydrate.

The results obtained according to the technique described in the proposed method are given in Table III.

TABLE III

	SS in 5 Gm. of Mixture, mg.	0.1 *M* $NaNO_2$ Equiv. to SS Present, ml.	0.1 *M* $NaNO_2$ Consumed, ml.	SS equiv. to 0.1 *M* $NaNO_2$ Consumed, mg.
Succinylsulfathiazole[a]	200	5.36	5.40	201.64
Succinylsulfathiazole and lactose[a]	200	5.36	5.39	201.26
Streptosuccinyl Cosmos[a]	200	5.36	5.35	199.76

[a] Average of 3 determinations in each case.

The technique described is based on the fact that the acylated sulfa drug is hydrolyzed in acid as well as in alkaline medium (2). In the former case there does not exist the danger of rupture of the heterocyclic ring at the nitrogen position provided dilute acid is used with a short heating period (HCl 12%, reflux an hour) (3).

The fact that in the U. S. P. XV determination of phthalylsulfathiazole (pp. 546–547) a prior acid hydrolysis is carried out is a corroborative instance of the method we describe. We have also noted recently that in the new supplement of the French Codex (4) an acid hydrolysis precedes the diazotization determination.

CONCLUSIONS

1. We have noted interferences in the determination of succinylsulfathiazole by the method described in the U. S. P. XV for this drug, particularly when lactose is also present.

2. An accurate determination of succinylsulfathiazole can be made in the presence of lactose by using a prior hydrolysis in acid medium according to the technique described.

3. The former considerations have a practical importance in determining succinylsulfathiazole in tablets containing lactose.

4. Conclusions 1, 2, and 3 indicate that succinylsulfathiazole can be estimated by diazotization when the preliminary hydrolysis is done in acid medium.

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Bacitracin III. Formulatory Characteristics of Zinc Bacitracin*

By HERBERT M. GROSS,† WILLIAM A. JOHNSON, and GRANT J. LAFFERTY

Zinc bacitracin was shown to be more stable than bacitracin in formulated troches, ointments, and tablets. Taste evaluations indicated zinc bacitracin to be less bitter than bacitracin and more easily masked.

ZINC BACITRACIN has been recognized as a stable form of bacitracin suitable for use in pharmaceuticals and animal feeds. A review of bacitracin literature (1) reveals several complexing and precipitating agents which yield active bacitracin compounds but none which possess all the desirable properties of zinc bacitracin. No other bacitracin compound has been accepted for human and veterinary use.

Zinc bacitracin was accepted as a certified antibiotic by the Food and Drug Administration in 1953. Since its original inclusion as section §146e.418 of the Federal Register, many other sections have been amended to include zinc

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bacitracin. It is now approved for use in ointments, tablets, implantation pellets, suppositories, and troches either alone or in combination with other microbiotics or therapeutic agents. Zinc bacitracin is not administered parenterally. These changes in the Federal Register result from the utilization of zinc bacitracin in bacitracin products because of the relative stability of this complex compared to bacitracin.

Due to the inherent stability of zinc bacitracin, it is felt that this complex will assume a role of importance relative to bacitracin similar to that which procaine penicillin holds in the penicillin field (2). A discussion of the physical, chemical, toxicologic, formulatory, and therapeutic properties of zinc bacitracin has previously appeared (2). This article reports the stability of bulk and formulated zinc bacitracin in comparison with bacitracin stability (3) and compares the tastes of zinc bacitracin and bacitracin.

EXPERIMENTAL

Stability of Bulk Zinc Bacitracin and Bacitracin.—Samples of zinc bacitracin and bacitracin were stored in sealed containers at 25°, 40°, and 60° and assayed for bacitracin activity after six, twelve, eighteen, and twenty-four months. The determinations were performed essentially by the U. S. P. XV microbiological cylinder-plate assay method for bacitracin. Results of these tests were recorded in Table I.

TABLE I.—THERMAL STABILITY OF ZINC BACITRACIN AND BACITRACIN BULK ANTIBIOTICS

Stored at:	Percentage Retained Bacitracin Activity					
	25°		40°		60°	
Time (months)	Zinc Bacitracin	Bacitracin	Zinc Bacitracin	Bacitracin	Zinc Bacitracin	Bacitracin
6	100	92	100	88	100	66
12	100	88	100	82	97	..
18	100	85	100	74	94	..
24	100	82	100	..	90	..

An additional experiment was conducted to determine the stability of zinc bacitracin and bacitracin at 105°. Test samples were taken at one-half, one, two, four, eight, and twenty-four hours and retained bacitracin activities determined. Results of these tests were recorded in Table II.

Stability of Zinc Bacitracin and Bacitracin in Troches.—Compressed tablet-type troches were prepared containing zinc bacitracin or bacitracin at a 1,000-unit per troche potency. The wet granulation method was used with ingredients similar to those reported by Bond, Himelick, and Macdonald (4) but with the inclusion of benzocaine as a local anesthetic. After original potency determinations, troches were stored at 25°, 40°, and 60° and reassayed for retained bacitracin activity after three, six, twelve, eighteen, and twenty-four months. Results of these tests were recorded in Table III.

TABLE II.—THERMAL STABILITY OF ZINC BACITRACIN AND BACITRACIN AT 105°

Time (hours)	Percentage Retained Bacitracin Activity	
	Zinc Bacitracin	Bacitracin
0.5	99.6	97.0
1	100.0	93.8
2	97.5	88.5
4	96.5	81.2
8	91.4	70.0
24	82.5	53.8

TABLE III.—THERMAL STABILITY OF ZINC BACITRACIN AND BACITRACIN IN TROCHES

Stored at:	Percentage Retained Bacitracin Activity					
	25°		40°		60°	
Time (months)	Zinc Bacitracin Troche	Bacitracin Troche	Zinc Bacitracin Troche	Bacitracin Troche	Zinc Bacitracin Troche	Bacitracin Troche
3	100	93	100	69	77	24
6	97	80	95	67	75	32
12	93	78	90	66	72	..
18	93	75	87	64	67	..
24	90	69	86	63	66	..

Stability of Zinc Bacitracin and Bacitracin Combined with Neomycin in Tablets and Ointments.—Compressed tablets were prepared containing zinc bacitracin or bacitracin and neomycin sulfate. Each tablet contained 2,500 units of bacitracin activity and 25 mg. neomycin base. Solvent-granulated bacitracin or zinc bacitracin was blended with starch-granulated neomycin sulfate, starch-granulated mannitol inert excipient, and stearate lubricant. Original potencies were determined and tablets were stored at 25°, 40°, and 60° for eighteen months and reassayed. Results were recorded in Table IV.

TABLE IV.—STABILITY OF ZINC BACITRACIN OR BACITRACIN-NEOMYCIN PRODUCTS AFTER EIGHTEEN MONTHS

Stored at:	Percentage Retained Bacitracin Activity			
	5°	25°	40°	60°
Tablets zinc bacitracin-neomycin	...	93	86	65
Tablets bacitracin-neomycin	...	85	71	36
Ointment zinc-bacitracin-neomycin	100	100	94	..
Ointment bacitracin-neomycin	97	90	60	..

Petrolatum base ointments were prepared containing zinc bacitracin or bacitracin in combination with neomycin sulfate. Each gram of ointment contained 500 units of bacitracin activity and 3.5 mg. neomycin base. The antibiotics were thoroughly dispersed in dehydrated petrolatum and finished in a roller-mill to provide uniformity. After original potency determinations, the ointments were stored

at 5°, 25°, and 40°, then reassayed for retained antibiotic activities after eighteen months. Results were recorded in Table IV.

Neomycin assays were performed essentially by the U. S. P. XV microbiological cylinder-plate assay method for neomycin sulfate.

TABLE V.—ZINC BACITRACIN AND BACITRACIN TASTE EVALUATION

Sample Concentration u./ml.	Percentage of Tasters Detecting Bitterness in Sweetened Solution			
	Initial Threshold			
	Zinc Bacitracin	Bacitracin	Zinc Bacitracin	Bacitracin
0.5	8.3	41.7	...	...
1.0	41.6	91.7	...	8.3
2.0	66.6	...	8.3	58.3
3.0	83.3	100.0	66.7	91.7
4.0	100.0	...	83.3	...
5.0	...	...	91.7	100.0
6.0	...	...	100.0	...

Comparative Zinc Bacitracin-Bacitracin Taste Evaluation.—Samples of comparable potency zinc bacitracin and bacitracin were accurately weighed and dissolved in distilled water at potency levels of 0.5, 1, 2, 3, 4, 5, and 6 units per ml. After allowing sufficient time for maximum solution of the bacitracins, the test solutions were filtered and coded.

Each of 12 tasters was asked to designate the sample of each sequential potency series in which bitterness was first detected. This method established the bitterness threshold (5) for zinc bacitracin and bacitracin. Distilled water controls were employed in each series to allow an estimation of the accuracy of the tasters. A parallel test was conducted using water sweetened with 5% sucrose to determine if the comparative thresholds of bitterness varied in the presence of a masking agent. Results of the taste panel evaluations were recorded in Table V.

DISCUSSION

The comparative study of zinc bacitracin and bacitracin as recorded in Table I points to the excellent activity stability of zinc bacitracin at room and elevated temperatures. This property is desirable in the manufacture and shipping of bacitracin products and for storage purposes. The more intensified, twenty-four hour thermal test at 105° confirmed the results of the long term study and indicated the utility of zinc bacitracin in formulations requiring heat. This thermal stability feature of zinc bacitracin points to its utility in the manufacture of pharmaceutical products requiring heated ointment or suppository bases, oven-drying of granulations, molten sugars for candy troches, and other products involving elevated temperature unit operations.

A typical bacitracin formulatory study comparing the stability of zinc bacitracin with bacitracin indicated the desirable characteristics of zinc bacitracin. After two years storage at 40°, the product still passed the Food and Drug Administration minimum potency requirement. This property obviates the inclusion of high percentage excess antibiotic, thereby reducing the cost of the product.

Bacitracin is commonly synergistically combined with other antibiotics such as neomycin (6). The studies of combinations of zinc bacitracin and bacitracin with neomycin sulfate point to the inherent stability characteristic of zinc bacitracin in both tablet and ointment formulations. Again, this desirable quality is advantageous in allowing the manufacture of stable products at lesser costs. For purposes of this comparative study, zinc bacitracin was solvent-granulated as is customary with bacitracin. However, this procedure is not necessary with zinc bacitracin for it may be granulated by the wet granulation method without undue potency loss.

Taste panel evaluations of the comparative bitterness of zinc bacitracin and bacitracin indicated zinc bacitracin was one-fifth as bitter as bacitracin at a 0.5-unit per milliliter level and about one-seventh as bitter as bacitracin at a 2.0-unit per milliliter level in the presence of sucrose as a sweetening agent. These parallel studies indicated that the taste of zinc bacitracin was more easily disguised than bacitracin and that the presence of a bitterness masking adjuvant increased the bitterness threshold ratio between the two substances.

SUMMARY

1. Zinc bacitracin was shown to be more stable than bacitracin at room and elevated temperatures. Zinc bacitracin may be used in formulations requiring heat processing.

2. Application of the greater formulatory stability of zinc bacitracin over bacitracin was demonstrated in a troche formulation as well as in tablet and ointment form combined with neomycin.

3. Zinc bacitracin was shown to be less bitter than bacitracin.

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The Kinetics of the Hydrolysis of Atropine*

By PETER ZVIRBLIS, IRVING SOCHOLITSKY,† and ALBERT A. KONDRITZER

A study has been carried out on the chemical kinetics of the hydroxyl ion catalyzed hydrolysis of atropine in solution. The mechanism of this hydrolysis has been shown to consist of two reactions, the effect of each being governed by the hydroxyl ion concentration. The temperature dependency of each reaction has been established, and equations have been proposed which enable the approximation of the half-life of atropine undergoing hydrolysis at constant temperature and constant hydroxyl ion concentration. Half-life values calculated by these equations have been shown to compare favorably with those obtained experimentally.

INSTABILITY of atropine in solution is due mainly to the hydrolytic reaction yielding tropine and tropic acid. Several investigators have reported atropine to be very stable at low *p*H, even at autoclave temperatures, but at higher *p*H values hydrolysis occurred and was enhanced by increased temperature and hydroxyl ion concentration (1–4). However, no attempt has been made to study the problem from a chemical kinetic viewpoint.

The object of this study is to establish a basis for predicting the rate of hydrolytic reactions of atropine in solution at various hydroxyl ion concentrations and temperatures. Studies were made at temperatures ranging from 25 to 60° and at hydroxyl ion concentrations covering roughly a million fold range, i. e., 10^{-1} to 10^{-7} *N*. Periodic quantitative determinations of the residual ester were made at a number of hydroxyl ion concentrations for each of the temperatures used. Specific velocity constants and activation energies were determined and these were used to derive an equation that appears to be valid for widely differing conditions of temperature and hydroxyl ion concentration.

THEORETICAL CONSIDERATION

The proton catalyzed hydrolysis of atropine is slow. We have found the reaction velocity constant to be approximately 3×10^{-4} min.$^{-1}$ in 0.1 *N* HCl. For the purpose of this investigation, it may be assumed that the hydroxyl ion catalyzed reaction is the mechanism responsible for hydrolysis of atropine in solution. Two hydrolytic pathways are possible for atropine because in solution it can occur as the free base or as the charged ion. For the purpose of this study, hydrolysis of the free base will be designated Reaction 1; that of the charged ion will be designated Reaction 2.

This study is based considerably on the theoretical concepts employed by Higuchi, *et al.* (5), in their study on procaine. Using the derivations of these authors, the overall rate of hydrolysis of atropine may be expressed as

$$\frac{d \ln a}{dt} = \frac{(OH^-)}{k_b + (OH^-)} [k_1(OH^-) + k_2 k_b] \quad (1)$$

where a = total drug concentration; k_1 = specific velocity constant for Reaction 1; k_2 = specific velocity constant for Reaction 2; and k_b = dissociation constant of the base.

The right side of Eq. 1 is a constant at any given hydroxyl ion concentration. If Reactions 1 and 2 are essentially responsible for the hydrolysis of atropine, the overall rate of hydrolysis would be first order with respect to drug concentration. Therefore, at any constant hydroxyl ion concentration the half-life of the drug would be expressed as follows:

$$\frac{1}{t_{1/2}} = \frac{(OH^-)}{k_b + (OH^-)} \left[\frac{k_1(OH^-) + k_2 k_b}{0.693}\right] \quad (2)$$

Equation 2 may be expressed logarithmically:

$$-\log t_{1/2} = \log (OH^-) - \log [k_b + (OH^-)] + \log \left[\frac{k_1(OH^-) + k_2 k_b}{0.693}\right] \quad (3)$$

At relatively high hydroxyl ion concentration the equation reduces to:

$$-\log t_{1/2} = \log (OH^-) + \log k_1/0.693 \quad (4)$$

and at low hydroxyl ion concentrations the equation simplifies to:

$$-\log t_{1/2} = \log (OH^-) + \log k_2/0.693. \quad (5)$$

Equations 4 and 5 indicate that at both ends of the *p*H scale the rate of hydrolysis would be directly proportional to the hydroxyl ion concentration. In the intermediate region of the *p*H scale there would be an area of nonproportionality.

EXPERIMENTAL

Method of Analysis.—The experimental method used in this study consisted of a series of ultraviolet spectrophotometric determinations of atropine solutions undergoing hydrolysis at constant temperatures. Atropine sulfate U. S. P., recrystallized twice from ethanol, was used; the melting point was 189 to 190°.

The ultraviolet absorption spectra of atropine is practically identical with that of its hydrolytic product, tropic acid; therefore, a separation of the two compounds before scanning the solutions was

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necessary. The absorption curve for atropine in acid solution is shown in Fig. 1. Absorption measurements were made with a Beckman Model DU Quartz Spectrophotometer at wavelengths of 256.5 and 262 mμ. The molar extinction coefficients at these wavelengths were found to be 198 and 149, respectively. The ratio of the absorbances at the two wavelengths, being constant over a wide range of drug concentration, served as a check on the analyses.

Buffer system.—The hydroxyl ion concentration of the solutions was maintained by using either buffers or an excess of strong base. The change of hydroxyl ion concentration during the experimental period was limited to 0.1 pOH unit.

Buffer systems used in this study are listed in Table I. The pH of each buffer was determined at the temperature of the run.

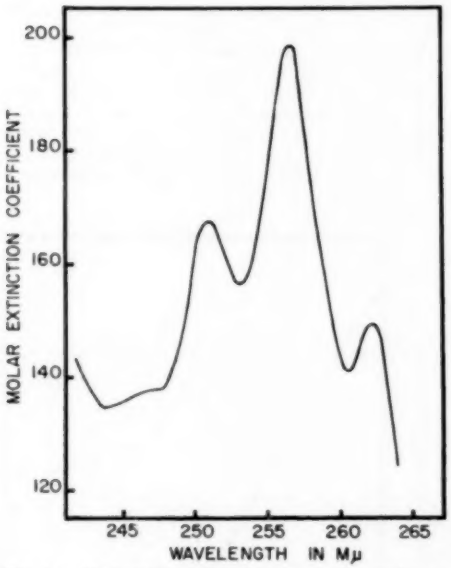

Fig. 1.—Ultraviolet absorption curve of atropine in 1.0 N HCl.

Experimental Procedure.—The procedure outlined below is essentially the one followed in this investigation.

Buffer solution was placed in a thermostatically controlled bath and allowed to reach temperature equilibrium. Sufficient standard solution, containing 10 mg./cc. of atropine sulfate, was then added to give in the reaction mixture a concentration of 1 mg./cc. The progress of the reaction mixture was followed by periodically removing 10-cc. samples for residual ester determination.

Tropic acid was separated from the residual ester by a modification of the U. S. P. method for purification of alkaloids (6). The sample for analysis was made alkaline with ammonium hydroxide; atropine, in base form, was extracted with two 10-cc. portions of chloroform. The chloroform extracts were combined, and atropine, in charged form, was then extracted with exactly 10 cc. of 0.1 N hydrochloric acid. The extent of hydrolysis was then calculated from the ultraviolet absorption of the acid extract.

TABLE I.—LIST OF BUFFERS

pH Range	Composition
6.99–7.97	$M/15$ KH_2PO_4 – Na_2HPO_4
8.03–9.89	$M/10$ H_3BO_3 + $M/10$ KCl + NaOH
10.01–10.51	$M/20$ $Na_2B_4O_7$ – Na_2CO_3

RESULTS AND DISCUSSION

Influence of Ester Concentration.—At constant temperature and hydroxyl ion concentration, the observed rate of hydrolysis of atropine is first order with respect to ester concentration. This is illustrated in Fig. 2, 3, and 4 representing, respectively, neutral, slightly alkaline, and moderately alkaline reaction conditions. These results are in accord with Eq. 1.

Influence of Hydroxyl Ion Concentration.—Figure 5 illustrates the effect of hydroxyl ion concentration on the hydrolytic reaction rate of atropine. The points were determined experimentally at 40°, whereas the smooth curve, which apparently fits the experimental points, represents Eq. 3. Values for k_1 and k_2 in this equation were obtained by substituting in Eq. 4 and 5, respectively, the extreme points of Fig. 5; the k_b for atropine was determined in 1% NaCl at 40°. These constants are: k_1 = 0.95 min.$^{-1}$; k_2 = 33.6 min.$^{-1}$; $k_b = 7.3 \times 10^{-5}$.

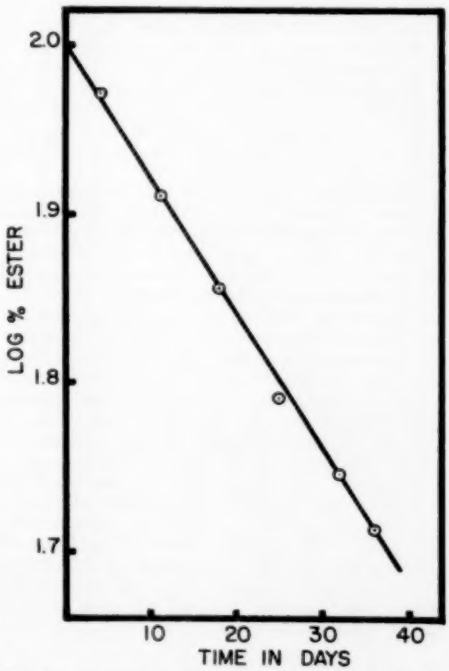

Fig. 2.—Hydrolysis of atropine in phosphate buffer at pH 7.10 and at 40°.

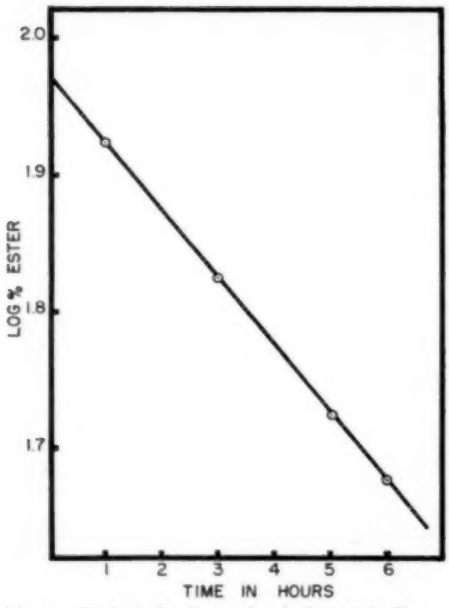

Fig. 3.—Hydrolysis of atropine in borate buffer at *p*H 9.89 and at 40°.

Figure 5 shows that above *p*H 11.5 the mechanism of hydrolysis is that of Reaction 1, or its equivalent; below *p*H 8.5 the mechanism is that of Reaction 2, or its equivalent. An area of nonproportionality lies between these two *p*H values.

The following study was carried out in order to determine the temperature dependency of Reactions 1 and 2, and to ascertain whether or not a change in the mechanism of hydrolysis of atropine is associated with a change in temperature.

Temperature Dependency of Reaction 1.—Velocity constants were determined at 25, 32.5, and 40° on three alkaline solutions of atropine that were 0.010 *N*, 0.020 *N*, and 0.040 *N* with respect to hydroxyl ion concentration. These measurements, which theoretically should represent a Type 1 reaction, were used for calculating the velocity constant at 1 *N* hydroxyl ion concentration for each of the temperatures used. In Fig. 6 the velocity constants are plotted against the reciprocal of the absolute temperature. The straight line relationship indicates that the mechanism responsible for the ester hydrolysis, essentially that of Reaction 1 or its equivalent, is not altered with changes in temperature. The slope of this line was calculated and by setting it equal to $-Ea/2.303\ R$, the energy of activation, *Ea*, was found to be approximately 7.7 Kcal. The frequency factor in the Arrhenius equation $k = Ze^{-Ea/RT}$ was found by substituting the conditions at 40°, i. e., $k_1 = 0.016$ sec.$^{-1}$ and $Ea = 7{,}700$ calories; *Z* comes out to be approximately 3.6×10^{2} sec.$^{-1}$,

$$\left.\begin{array}{ll} \text{or} & k_1 = 3.6 \times 10^{3}\, e^{-7{,}700/RT}\ \text{sec.}^{-1} \\ \text{and} & k_1 = 2.1 \times 10^{6}\, e^{-7{,}700/RT}\ \text{min.}^{-1} \end{array}\right\} \quad (6)$$

TABLE II.—EXPERIMENTAL HALF-LIVES OF ATROPINE AT HIGH *p*H COMPARED WITH PREDICTED VALUES

Temp.	*p*OH	Half-Life in Minutes By Experiment	Half-Life in Minutes By Equation
25.0	2.00	128.0	137.0
	1.70	63.6	68.3
	1.40	34.7	34.1
32.5	2.00	92.0	100.0
	1.70	46.2	49.8
	1.40	26.0	24.9
40.0	2.00	66.3	73.8
	1.70	34.1	36.7
	1.40	18.7	18.4

On substituting the value for Eq. 6 in Eq. 4 a formula is obtained for estimating the half-life of atropine above *p*H 11.5 at constant temperature and hydroxyl ion concentration. A comparison of the experimental values and those predicted by formula for Reaction 1 is given in Table II.

Temperature Dependency of Reaction 2.—Half-lives were determined at 40, 49.8, and 59.8° on solutions of atropine of *p*H 7.0, 7.5, and 8.0, since Fig. 5 indicates Reaction 2 to be the mechanism responsible for the hydrolytic reaction at these *p*H values. In Fig. 7 the logs of these half-lives are plotted against the reciprocal of the absolute tem-

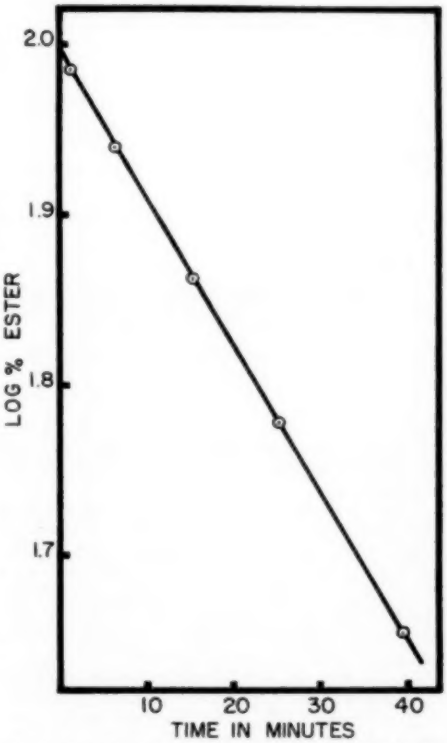

Fig. 4.—Hydrolysis of atropine in 0.02 *N* $Ba(OH)_2$ and at 40°.

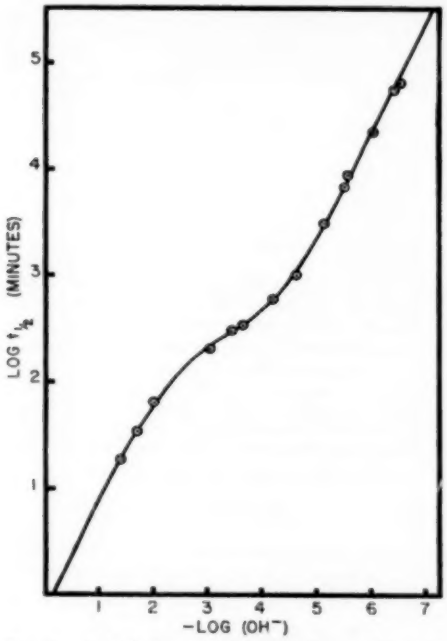

Fig. 5.—A Relation of half-life of atropine and hydroxyl ion concentration at 40°.

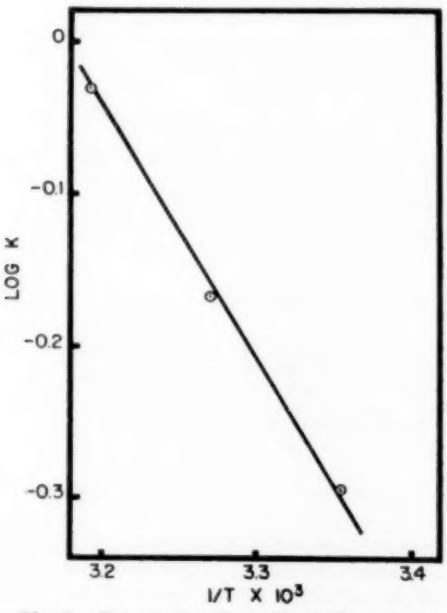

Fig. 6.—Temperature dependency of reaction 1. Log K min.$^{-1}$ *vs.* reciprocal of the absolute temperature of the run.

perature, $1/T$; from the slope of the lines the activation energy, Ea, was calculated. The experimental data, when subjected to an analyses of covariance (7), gave an average slope of 5,400, with a standard error of 250. The activation energy, calculated from the slope, comes out to be 24.7 ± 1.1 Kcal. Taking into account the heat of ionization of water, which is approximately 12 Kcal. at 40°, the activation energy for Reaction 2 is 24.7 minus 12, or 12.7 Kcal. per mole.

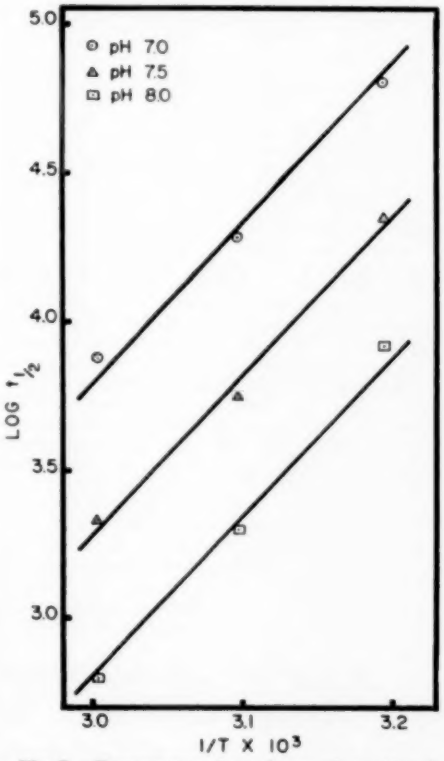

Fig. 7.—Temperature dependency of reaction 2. Log $t_{1/2}$ in minutes *vs.* reciprocal of the absolute temperature of the run.

The corresponding Arrhenius frequency factor for Reaction 2, when calculated from values for 40°, i. e., $k_2 = 0.56$ sec.$^{-1}$ and $Ea = 12{,}700$ calories comes out to be 3.5×10^8 sec.$^{-1}$,

$$\left.\begin{array}{ll} \text{or} & k_2 = 3.5 \times 10^8\, e^{-12{,}700/RT}\,\text{sec.}^{-1} \\ \text{and} & k_2 = 2.1 \times 10^{10}\, e^{-12{,}700/RT}\,\text{min.}^{-1} \end{array}\right\} \quad (7)$$

When the value for Eq. 7 is substituted in Eq. 5, one obtains a simple relationship that expresses the half-life of atropine solutions buffered below *p*H 8.5 as a function of *p*OH and temperature. In Table III half-lives of atropine solutions determined experimentally are compared with those predicted by Eq. 5.

The complete formula for the hydroxyl ion catalyzed hydrolysis of atropine may be obtained by substituting Eq. 6 and 7 into Eq. 3, i. e.

$$-\log t/_0 = \log (OH^-) - \log [7.3 \times 10^{-6} + (OH^-)] + \log [3.1 \times 10^5 e^{-7,700/RT} (OH^-) + 2.2 \times 10^5 e^{-12,700/RT}]$$

TABLE III.—EXPERIMENTAL HALF-LIVES OF ATROPINE AT NEUTRAL *p*H COMPARED WITH PREDICTED VALUES

Temp.	*p*H	Half-Life in Hours By Experiment	Half-Life in Hours By Equation
40.0	7.01	1044.0	1165.0
	7.10	906.0	947.0
	7.50	377.0	377.0
	7.97	149.0	128.0
49.8	7.01	315.0	346.0
	7.49	95.0	115.0
	7.94	38.4	40.7
59.8	6.99	128.0	110.0
	7.47	37.5	36.8
	7.97	11.3	11.6

This formula can be used to calculate the half-life in minutes of atropine solutions falling within the nonproportional section of the hydrolysis curve. Experimental half-lives of atropine in solution covering this range of *p*OH at 40° are compared with those calculated by this formula in Table IV.

This work has been concerned only with the hydrolysis of atropine in solution catalyzed by hydroxyl ions. Although the proton catalyzed hydrolysis of atropine is very slow by comparison, it does exist and indications are that it may become of practical significance at a *p*H value of about 4 and lower. A kinetic study of the proton-catalyzed reaction is presently being carried out in this laboratory. Data which will be obtained from this study, along with those presently reported, should provide a basis for predicting the *p*H range over which atropine in solution is least prone to the overall effect of both hydroxyl ion and proton-catalyzed hydrolysis, i. e., most stable during prolonged storage.

TABLE IV.—EXPERIMENTAL HALF-LIVES OF ATROPINE AT MODERATE *p*H AND 40° COMPARED WITH PREDICTED VALUES

*p*H	Half-Life in Hours By Experiment	Half-Life in Hours By Equation
8.03	114.0	116.0
8.37	50.8	55.4
8.91	16.1	19.2
9.34	10.0	9.9
9.89	5.8	5.7
10.01	5.1	5.0
10.51	3.4	3.7

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Assay of 2-Aminoheptane and Methyl Hexane Amine in Plastic Inhalers by Titration in Glacial Acetic Acid*

By J. P. COMER and E. E. KENNEDY†

The 2-aminoheptane and methyl hexane amine content of plastic inhalers can be determined rapidly and accurately by titrimetric assay in glacial acetic acid solution. The amine is extracted directly from the saturated pledget with glacial acetic acid and titrated with perchloric acid. 4-Aminopyridine was found to offer advantages over potassium acid phthalate for the standardization of the perchloric acid-glacial acetic acid solution.

THE ASSAY of 2-aminoheptane[1] and methyl hexane amine[2] in plastic inhalers by the usual aqueous acid extraction and titration is made difficult by two factors. The amine is so strongly adsorbed on the cotton pledget that it requires a prolonged extraction on the steam bath in acid solution to completely extract the amine. The hot acid extraction also has been found to remove basic constituents from the plastic inhaler. This makes it necessary to perform a blank titra-

* Received January 19, 1956, from Eli Lilly and Company, Indianapolis 6, Ind.
† Analytical Department, Eli Lilly and Co., Indianapolis 6, Ind.
[1] Inhaler Tuamine® (tuaminoheptane, Lilly).
[2] Inhaler Forthane® (methyl hexane amine, Lilly).

tion on the empty inhaler. It was the object of the work reported here to develop a rapid and accurate method which would not require the blank determination.

Chatten and Pernarowski (1) titrated ephedrine in oily and aqueous nasal sprays in nonaqueous solvents. The oily solution of the aminoheptanes used in the plastic inhalers is readily soluble in chloroform and acetic acid so the use of titration in these solvents offered a very logical approach to the assay of the contents of these inhalers. A method utilizing acetic acid as the solvent, chloroform as the washing solution, and 0.2 *N* perchloric acid as the titrant was found to be satisfactory for the routine assay of the inhalers containing these compounds.

4-Aminopyridine was shown by Stone and Van Hall (2) to be a useful primary standard in aqueous systems, and it was found here to offer advantages over potassium phthalate for standardization of perchloric acid since the amine is more soluble in glacial acetic acid.

EXPERIMENTAL

The volatility of the aminoheptanes makes it necessary to rapidly transfer the amine-saturated pledget from the sealed inhaler into the acetic acid solution. Ideally, the tightly packed pledget should be cut with a sharp knife beneath the acetic acid solution, but it was found that the loss of the amine was negligible when the pledget was cut on glassine paper held in the hand. The glassine paper used in this laboratory does not give a blank titration, and it may be placed with the pledget into the glacial acetic acid solution. When the slit pledget is swirled by the stirring rod, the amine is exhaustively extracted by the time the titration is completed. After the end point is reached, an additional minute of stirring will verify the completeness of extraction if the indicator color does not change. With the exception of the preparation of the reagents, the assay can be completed in less than ten minutes.

TABLE I.—RECOVERY OF 2-AMINOHEPTANE FROM HAND-FILLED INHALERS

Inhaler	Found, mg.
A	337
B	335
C	338
D	339
E	338
Theory	338

The accuracy of the method is illustrated by the recovery data in Table I. In this experiment, a known amount of 2-aminoheptane was added manually with a syringe to the cotton in the blank inhalers. The results seen in Table II are representative of routine assays of machine-filled inhalers.

TABLE II.—RESULTS FROM ROUTINE ASSAYS OF MACHINE-FILLED INHALERS

Inhaler	2-Aminoheptane, mg.	Methyl Hexane Amine, mg.
A	351	254
B	351	258
C	353	256
D	352	254
E	353	250
Labeled amount =	325	Labeled amount = 250

REAGENTS

Crystal Violet Indicator 2%.—Dissolve one Gm. of crystal violet in 50 ml. of glacial acetic acid.

Perchloric Acid 0.2 *N*.—To 500 ml. of glacial acetic acid in a 1000-ml. volumetric flask, add 17.0 ml. of 70% perchloric acid. Mix, and then add sufficient glacial acetic acid to make 1000 ml. of solution and mix again. Dissolve 0.200 Gm. of 4-aminopyridine in 100 ml. of glacial acetic acid. Add one drop of crystal violet indicator, titrate with the perchloric acid solution, and calculate the normality.

METHOD

Open the plastic inhaler with the aid of clean pliers and immediately slit the cotton pledget with a clean knife while holding the pledget in a small piece of glassine paper. Place the pledget and the glassine paper in 100 ml. of glacial acetic acid, and then rinse the inside of the inhaler and the knife several times with chloroform. Press the pledget with a glass stirring rod to squeeze out the oily base and stir in such a manner that the slit pledget will revolve with the stirring rod. Add one drop of crystal violet indicator and titrate with 0.2 *N* perchloric acid to the same end point used for the standardization of the perchloric acid. Each ml. of 0.2 *N* perchloric acid is equivalent to 23.0 mg. of 2-aminoheptane or methyl hexane amine.

SUMMARY

The 2-aminoheptane or methyl hexane amine content of plastic inhalers may be conveniently determined by titration in glacial acetic acid. The amine-saturated pledget is extracted directly in the glacial acetic acid titration solvent and titrated with 0.2 *N* perchloric acid. The method is rapid, sufficiently accurate, and no blank titration is required. Because of the ready solubility of 4-aminopyridine in glacial acetic acid, this amine was found to have advantages over potassium acid phthalate for the standardization of perchloric acid.

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A New Preparative Cell for High Voltage Electrophoresis*

By RUPERT SALISBURY† and E. P. GUTH‡

A new electrophoresis cell has been designed in an attempt to effect separation of substances. A separation of Pharmagels A and B was achieved. Four mixtures of known, pure ingredients were subjected to electrophoresis in this new cell and in each case partial separation was achieved. The separation of two plant extracts was also carried out.

ELECTROPHORESIS has long been used both as an analytical and as a preparative method for the investigation of colloids. Some investigation has been made of the electrophoretic behavior of simple electrolytes primarily as a means for determining transference numbers by the moving boundary method. The majority of compounds of pharmaceutical interest fall into a molecular weight range somewhere between simple electrolytes and colloids. Materials in this intermediate range for the most part obtain a charge by simple ionization. The application of electrophoresis to this class of substances has been hampered by the minute amounts of material used and the stringent conditions necessary for the moving boundary technique.

In 1929, Williams and Waterman (1) reported the use of a 12-compartment dialysis cell as a means of characterizing ampholytes. When a 110-volt d.-c. potential was applied to an aqueous system, a *p*H gradient was established between the electrodes due to the migration of ions and cations present in the solution. An ampholyte would migrate to that section of the system where the *p*H would approximate its isoelectric point and become a neutral ion. Electrolytes, in contrast, would ionize, and the ions would migrate to the electrode of opposite charge.

Williams and Truesdail (2) showed that this principle could be used to isolate constituents of rice bran extract. Their apparatus which utilized cylinders connected by siphons was further modified by these and other workers (3–10).

In these cases the potential varied from 110 to 11,000 volts d. c. and the current from 10 microamperes to 20 milliamperes. Without exception, the connections between compartments, designed to permit isolation of the individual segments of the cell, were smaller in area than the segments themselves. This caused eddy currents within each segment compartment and resulted in a variable field strength within the segment. Since the rate of migration of a charged particle is a function of field strength, this led to a varied migration rate for identical particles in different parts of the same segment and thus opposed separation.

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Abstract of a dissertation submitted to the Graduate School of the Ohio State University by Rupert Salisbury in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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EXPERIMENTAL

The Preparative Cell

The cell used in this study consisted of 5 "V"-tubes of 1.75 cm. i. d., having arms of approximately 12 cm. in length. These were joined together and equipped with stopcocks and provided with air vents (Fig. 1). In this cell, the main lines of the electrical field will be uniformly parallel throughout the linear length of approximately 100 cm. The stopcocks permit drainage of each "V," thus converting the cell into five segments and permitting fractionation of the sample. The entire cell has a capacity of about 200 ml.

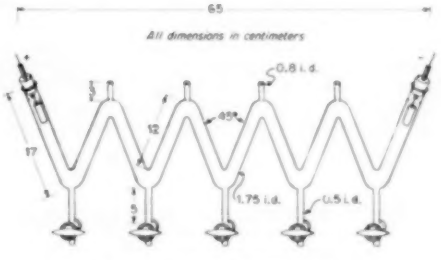

Fig. 1.—Electrophoresis cell.

The electrodes consisted of heavy platinum foil, $1/2 \times 1$ inch, welded to 6-inch lengths of platinum wire. The leads were encased in glass tubing and sealed at the electrode end. The platinum foil was curved to fit into the tubular electrode vessels. The electrodes were held in place by means of slotted corks to permit escape of gaseous products. The electrodes were placed just below the surface of the solutions in the terminal sections. The cell was held level by means of steel supports.

The material to be studied was usually deposited

TABLE I.—FORMATION OF THE *p*H GRADIENT WITH DISTILLED WATER

Time Interval, hr.	Current, ma.	Segment I (anode)	II	III	IV	V (cathode)
0.0	1.2	6.7	6.7	6.7	6.7	6.7
0.1	0.9	5.8	6.2	6.5	6.5	8.6
0.3	0.6	4.9	6.0	6.2	8.9	9.4
0.5	0.5	5.5	5.6	5.7	9.2	9.4
1.0	0.4	5.2	5.6	5.6	5.9	9.5
2.0	0.1	5.2	5.6	5.8	6.3	9.7
16.0	0.1	5.4	6.2	6.6	6.0	9.1

at the bottom of segment III by means of a length of polyvinyl tubing attached to a hypodermic syringe, and inserted through the air vent. Mixing of the sample with the solvent in the cell was avoided. This procedure caused a portion of the sample to remain in the neck of the drain tube beyond the reach of the electrical field. This portion was removed before collecting the cell contents.

The rectifier used[1] was capable of supplying from 1 to 15,000 volts of d. c. at 0.5 ampere.

Use of the *p*H Gradient in Separation of Ampholytes

Formation of the *p*H Gradient.—*Distilled Water.*—The cell was filled with distilled water, and a 15,000-volt potential applied. The current ranged from 0.1 to 1.4 milliamperes and the temperature from 30 to 50°. At intervals the entire cell was drained, and the *p*H of each fraction was determined, using a Beckman Model C *p*H meter with a glass electrode. These *p*H values represented a mean for each segment; the actual *p*H values are present in a continuous gradient throughout the segment (Table I). The *p*H of the distilled water was found to be 6.7.

N/1,000 NaOH.—The cell was filled with *N*/1,000 NaOH, and periodic *p*H determinations were made as with distilled water to determine the extent to which the *p*H gradient may be shifted. Due to the greater conductivity of the solution, the potential applied was 1,000 volts. The current ranged from 0.35 to 2.5 milliamperes and the temperature from 30 to 35° (Table II).

Application to Proteins.—Pharmagels A and B (11) were selected for separation since they are chemically almost identical, differing only in isoelectric points. Pharmagel A has its isoelectric point at *p*H 8, while Pharmagel B has an isoelectric point at about 4.7. The qualitative test used for gelatin was the turbidity caused by tannic acid test solution.

(*a*) Four milliliters of a 1% solution of pharmagel A in distilled water was placed at the bottom of segment III, and a 5,000-volt potential was applied for one hour.

	I	II	III	IV	V
Results:					
*p*H	3.6	4.8	5.2	6.4	9.8
Gelatin	−	−	−	+	+

(Pharmagel A migrated from segment III into segments IV and V.)

(*b*) Four ml. of a 1% solution of pharmagel B in distilled water was treated as above.

	I	II	III	IV	V
Results:					
*p*H	3.8	5.2	5.2	7.0	10.0
Gelatin	−	+	+	−	−

(Pharmagel B migrated from segment III into II.)

(*c*) A mixture consisting of 4 ml. each of 1% solutions of pharmagels A and B was placed at the bottom of segment III, and a 6,000-volt potential was applied for two hours.

	I	II	III	IV	V
Results:					
*p*H	3.3	4.0	6.4	9.0	10.4
Gelatin	−	−	+	+	−

(Pharmagel A in segment IV; pharmagel B in segment III.)

TABLE II.—FORMATION OF THE *p*H GRADIENT WITH *N*/1,000 NaOH

Time Interval, hr.	Current, ma.	Segment I (anode)	II	III	IV	V (cathode)
0.0	2.5	10.3	10.3	10.3	10.3	10.3
0.3	2.2	10.4	10.5	10.5	10.5	10.8
0.5	2.0	10.2	10.5	10.6	10.7	10.9
1.0	1.5	9.8	10.6	10.6	10.6	11.0
2.0	1.0	8.8	10.6	10.7	10.6	11.1
10.0	0.4	5.5	9.4	10.6	10.8	11.3

(*d*) An attempt was made to extend the separation of the two Pharmagels by use of *N*/10,000 NaOH instead of distilled water. Three milliliters each of 1% solutions of pharmagel A and B in distilled water were placed at the bottom of segment III after filling the cell with *N*/10,000 NaOH, and a 6,000-volt potential was applied for two hours, and

[1] Army Surplus Rectifier (RA 38).

then a 15,000-volt potential was applied for two more hours.

	I	II	III	IV	V
Results:					
*p*H	2.9	5.0	6.1	9.2	11.0
Gelatin	−	+	−	+	−

(Pharmagel A in segment IV; Pharmagel B in segment II.) A separation of Pharmagel A from B was achieved.

Migration Rate Differential

Separation of Organic Mixtures.—*Acid Dyes.*—FD&C Blue #1 and Yellow #5 were selected because of their known acidity and so that the degree of separation could be followed visually.

One milliliter of a mixture of 1% each FD&C #1 and Yellow #5 in distilled water was placed in segment V after the cell had been filled with distilled water. A 15,000-volt potential was applied for one and one-half hours. In this case, since the separation was visible, the fractions were all removed from segment I (the anode). The first fraction (Ia), a clear yellow, was removed from segment I after twenty minutes. Fraction Ib, a yellow-green, was removed fifteen minutes later (a total elapsed time of thirty-five minutes). Fraction Ic, a kelly green, was removed after a total elapsed time of one hour. Fraction Id, a blue-green, was removed after a total elapsed time of ninety minutes. Thus a separation was achieved, resulting in a pure blue, a pure yellow, and three mixtures.

Strychnine-Brucine.—Five milliliters of a solution containing 0.05 *N* each strychnine and brucine gluconate in distilled water was placed at the bottom of segment II. Fractions were removed from segment V at time intervals shown in Table III. After a total elapsed electrophoresis time of twenty-six hours and thirty-six minutes, the final five fractions were obtained. Identification of the presence of alkaloids in the fractions was made with Valser's reagent (12). The aqueous solutions were extracted with chloroform following standard procedures. The chloroform residue was dissolved in a few drops of diluted HCl, and specific identification tests for the alkaloid present were made. The microscopic tests used were the mercuric chloride T. S., crystal formation with brucine and the crystals formed by strychnine upon the addition of a drop of platinic chloride T. S. (13). Confirmatory tests were made upon the extracted alkaloids by the addition of a drop of nitric acid (13). Results: The first five fractions removed contained strychnine, but no brucine. Brucine did not appear in admixture until the 6th fraction. All other fractions, including the five final fractions obtained by draining the entire cell after a total elapsed time of twenty-six and one-half hours, contained both brucine and strychnine.

Morphine-Codeine.—Five milliliters of a solution containing 1% each morphine and codeine sulfates in distilled water was placed at the bottom of segment II. Time intervals of fractionation, voltage, and other conditions are tabulated in Table IV. Identification tests for morphine and codeine were the microscopic examinations of crystal types formed with Marmé's T. S. (13). The alkaloids were extracted from the aqueous solutions by standard procedures using chloroform. The chloroform residue was dissolved in a few drops of diluted HCl. A few drops of this solution were then treated with a drop of Marmé's T. S. and the resulting crystal forms examined microscopically. Results: Nine fractions were removed and found to contain only codeine. No morphine was found until the 10th fraction was collected.

Salicylic Acid-Benzoic Acid.—Five milliliters of a 0.1 *N* solution of sodium salicylate and sodium benzoate in distilled water was placed in segment IV. Time intervals, voltage, and other conditions are shown in Table V. The conductivity of this system was high, and the applied potential was low by comparison, being stabilized at 3,000 volts after the initial period.

The acidified fractions were extracted with several portions of ether, the ether portions were combined and evaporated, and the residue was dissolved in a few drops of 2% triethanolamine solution. A few drops of this solution were treated with silver nitrate T. S., and the resulting crystal forms examined microscopically (14). Results: Neither acid was present in the first six fractions. The 7th to the 13th

TABLE III.—RESULTS OF THE STRYCHNINE-BRUCINE SEPARATION

Time Interval, hr.	Fraction	Voltage	Current, ma.	Temperature, °C.	*p*H	Valser's Reagent	Brucine	Strychnine	Nitric Acid
1.0	V a	10,000	1.9	52	9.55	+	−	+	Yellow
2.5	V b	10,000	1.9	54	9.5	+	−	+	Yellow
4.0	V c	10,000	2.5	59	10.0	+	−	+	Yellow
5.0	V d	10,000	3.0	59	9.4	+	−	+	Yellow
6.0	V e	10,000	3.5	67	10.0	+	−	+	Yellow
18.0	V f	3,000	0.55	32	10.0	+	+	+	Red
19.5	V g	10,000	5.0	75	9.9	+	+	+	Red
20.5	V h[a]	5,000	0.95	46	9.2	+	+	+	Red
22.5	V i	7,000	2.3	46	9.3	+	+	+	Red
25.5	V j	7,000	2.3	70	9.5	+	+	+	Red
26.5	V k	7,000	1.75	60	9.3	+	+	+	Red
26.5	IV	...	...	...	9.0	+	+	+	Red
26.5	III	...	...	...	9.5	+	+	Trace	Red
26.5	II	...	...	...	3.1	+	+	Trace	Red
26.5	I	...	...	...	2.5	+	Trace	Trace	No color

[a] Crystals were found on the walls of segment V when fraction V h was removed. These crystals were identified as a mixture of strychnine and brucine.

the opium apparently masked the specific test for charge on the strychnine molecule greater than that

TABLE IV.—RESULTS OF THE MORPHINE-CODEINE SEPARATION

Time Interval, hr.	Fraction	Voltage	Current, ma.	Temperature, ° C.	pH	Chloroform Extract		Aqueous Extract,
						Morphine	Codeine	Morphine
0.5	V a	10,000	1.5	31	9.75	−	+	−
1.0	V b	10,000	1.15	42	9.0	−	+	−
2.0	V c	10,000	0.8	47	9.0	−	+	−
3.0	V d	10,000	0.8	47	9.3	−	+	−
4.0	V e	10,000	0.9	50	9.2	−	+	−
5.0	V f	10,000	0.93	49	9.0	−	+	−
6.0	V g	10,000	1.1	52	9.1	−	+	−
15.0	V h	10,000	3.3	72	9.5	−	+	Trace
20.0	V i	10,000	0.95	40	9.0	−	+	Trace
20.5	V j	10,000	1.3	56	8.7	+	+	+
20.5	IV	...	...	...	8.65	+	+	+
20.5	III	...	...	...	8.6	+	−	+
20.5	II	...	...	...	8.15	+	−	+
20.5	I	...	...	...	2.3	−	−	−

fractions inclusive contained salicylic acid but no benzoic acid. All remaining fractions, from the 14th on, contained both acids.

Separation of medicinal Plant Extracts.—*Nux Vomica Tincture.*—Since previous experiments with the pure alkaloids had shown that it was necessary to maintain the initial solution as nearly neutral as possible, the crude tincture was treated in the following manner. Twenty-five ml. of Nux Vomica Tincture was diluted to 50 ml. with distilled water, and the mixture made neutral with diluted ammonia solution. The resulting mixture was further diluted to 60 ml. with 50% gluconic acid solution.

Since Nux Vomica Tincture contains about 0.1% strychnine, the 20 ml. of the diluted tincture used represents only $^1/_{30}$ the amount of strychnine present in the 5 ml. of the 0.1 *N* solution used previously.

Twenty milliliters of the prepared Nux Vomica Tincture was placed in segment III. Results comparable to the known mixture were obtained. All the alkaloidal fractions were clear and colorless. The colored extractive material and all colloidal material present in the original tincture were apparently negatively charged and migrated to the anode.

Opium Tincture.—Ten grams of powdered opium was extracted with boiling distilled water. The extract was evaporated to 100 ml. in a current of warm dry air and clarified by centrifugation. Twenty milliliters of the resulting opium tincture was placed in segment III and subjected to electrophoresis.

The fractions obtained were tested for alkaloids in the same manner as the morphine-codeine mixture. Tests were also made for the presence of meconic acid (15).

No positive tests for morphine or codeine were obtained. The large variety of alkaloids present in

TABLE V.—RESULTS OF THE SALICYLIC-BENZOIC ACID SEPARATION

Time Interval, hr.	Fraction	Voltage	Current, ma.	Temperature, ° C.	pH	Benzoic Acid, Silver Nitrate	Salicylic Acid	
							Silver Nitrate	Ferric Chloride
0.33	I a	5,000	0.37	31	6.6	−	−	−
0.66	I b	5,000	0.67	31	5.2	−	−	−
1.25	I c	3,000	0.55	30	5.2	−	−	−
1.75	I d	3,000	0.7	30	5.1	−	−	−
3.0	I e	3,000	0.8	30	5.1	−	−	−
4.0	I f	3,000	0.7	30	5.6	−	−	−
5.0	I g	3,000	1.2	37	4.4	−	+	+
6.0	I h	3,000	1.2	39	4.4	−	+	+
7.0	I i	3,000	1.35	40	4.3	−	+	+
8.25	I j	3,000	1.4	42	4.3	−	+	+
9.5	I k	3,000	1.7	46	4.2	−	+	+
11.5	I l	3,000	2.0	48	3.95	−	+	+
12.75	I m	3,000	2.0	49	3.95	−	+	+
24.75	I n	3,000	1.3	30	3.4	+	+	+
25.0	I o	3,000	3.0	47	3.7	+	+	+
27.0	I p	3,000	3.5	48	3.8	+	+	+
28.25	I q	3,000	3.0	55	3.7	+	+	+
29.75	I r	3,000	2.9	48	3.7	+	+	+
32.0	I s	3,000	4.0	57	3.5	+	+	+
33.5	I t	3,000	3.0	57	3.7	+	+	+
35.5	I u	3,000	4.0	58	3.7	+	+	+
35.5	II	...	...	...	3.9	+	+	+
35.5	III	...	...	...	3.9	+	+	+
35.5	IV	...	...	...	4.5	+	Trace	+
35.5	V	...	...	...	11.7	−	−	−

the opium apparently masked the specific test for these two alkaloids. Since it was not possible to isolate morphine and codeine, the alkaloid-containing fractions were simply identified with the general alkaloidal precipitant, Valser's reagent. Results: The complex mixture of alkaloids was present in each of the fractions collected from the cathode and in segments II, III, and IV when the cell was finally drained after thirty-one hours of electrophoresis.

Meconic acid was found present only in a fraction removed from the anode after twenty-nine hours of electrophoresis and in the anode segment (I) when the experiment was concluded. All alkaloidal fractions were clear solutions. All colored extractive material and colloidal matter migrated to the anode.

DISCUSSION

The separation of ampholytes has been shown to be possible, provided the *p*H gradient in the cell is such that the isoelectric points are found in different segments of the cell. The *p*H gradient may be altered by the use of dilute acids and bases so that a gradual *p*H gradient over a short *p*H range may be obtained.

The migration rate through one "V" segment has a mean value close to that of a straight tube. Larger cells, higher potentials, or longer electrophoresis times will handle proportionately larger amounts of material. A cooling bath will be necessary at higher current densities.

The successful separation of two materials appears to be a function of both the ionization constants and the rates of migration of the materials involved. In order for migration to occur, ionization must take place. Once the ions are present, then the separation becomes a question of the comparative rates of migration.

The speed at which a substance will move through the cell will be determined by the net charge per unit volume, the shape of the migrating unit (in these cases the ion), and the strength of the electrical field. The variation of migration speed in two of the mixtures investigated is readily understood. Morphine differs from codeine only in that morphine contains a free phenolic hydroxyl group while codeine contains a methoxyl group in the same position. Thus, the positive charge on the morphine molecule due to the amine function would be negated in part by the smaller negative charge due to the phenolic hydroxyl group. Since the methoxyl group on the codeine molecule is nonionogenic, it contributes nothing in the way of a charge, and the net charge upon the morphine molecule will be less than the net charge upon the codeine molecule.

In the case of the benzoic acid-salicyclic acid mixture, it was found that the phenolic hydroxyl group acted in support of the total negative charge on the salicylic acid molecule. The negative charge due to the ionization of the phenolic hydroxyl group must be added to the negative charge due to the ionization of the carboxyl group so that the salicylic acid molecule has a greater charge than the benzoic acid molecule and will migrate more rapidly.

The only difference between strychnine and brucine is the additional pair of methoxyl groups on the brucine molecule. This would tend to make the net charge on the strychnine molecule greater than that upon the brucine molecule on a weight basis and cause a more rapid migration rate for the strychnine molecule.

The separation of crude plant extracts as indicated by the work on Nux Vomica Tincture and Opium Tincture shows promise. Colloidal material can be completely eliminated where the constituents of interest are positively charged. The two extracts tested were of this type and the alkaloids were obtained free from admixture with colloidal matter and coloring matter in clear, colorless solutions.

One of the factors which will limit the use of this technique is the possible decomposition of experimental material, either due to the *p*H gradient of the cell or decomposition of the material at the electrodes. Electrode reactions of complex organic materials have not been thoroughly studied, and it would be difficult to predict electrode decomposition.

It has been shown that the technique demonstrated is capable of separating constituents in the few mixtures investigated. Additional work remains to be done to refine this technique, but the method does show promise.

SUMMARY

1. A new fractional electrophoresis cell has been designed for use in either the high voltage or in the moving boundary method.

2. This cell has been used to separate Pharmagel A from Pharmagel B.

3. Mixtures of strychnine-brucine, morphine-codeine, salicylic acid-benzoic acid, and FD&C Blue #1 and Yellow #5 were subjected to electrophoresis and partial separations were achieved.

4. An alkaloidal fraction was separated from nux vomica tincture, and pure strychnine could be separated from the mixture. Opium tincture, due to its rather high content of various alkaloids, did not lend itself to a separation of the alkaloids. The mixture of alkaloids was, however, separated from the rest of the tincture.

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active alkylating functions, such as triethylenemela- cyanuric chloride could not be replaced by weak

Amine Derivatives of Cyanuric Chloride II. Amino Esters*

By WILLIAM O. FOYE† and LESTER CHAFETZ‡

Cyanuric chloride has been condensed with glycine and β-alanine esters to give a series of amino ester derivatives of the s-triazine ring. 2,4,6-Tris-(carbethoxyalkyl) derivatives could not be formed in aqueous media, nor could condensation with urethan be effected. Biological activities of these and other triazines are described.

THE FIRST PAPER of this series (1) described the preparation of a number of s-triazines containing such pharmacologically active amine substituents as the 2-aminothiazole, 2-aminopyridine, β-diethylaminoethylamine, and 2,4′-diamino-5-thiazolylphenylsulfone (Promizole®) groups. A variety of biological tests has been carried out on these compounds, and it is the purpose of this report to state the results of these tests, along with the preparation of another group of amino triazines which seemed justified.

DISCUSSION

In vitro antitubercular tests on several of the amino triazines showed only partial or no inhibition of growth of *M. tuberculosis* H37 Rv, but one of the compounds, the tris-(β-diethylaminoethylamino) derivative, showed positive *in vivo* activity when tested in mice. The latter compound also displayed growth inhibition of *E. histolytica in vitro*, its activity being about one-fourth that of carbarsone oxide. The antitubercular results are shown in Tables I and II. The low *in vitro* antitubercular activity displayed by these compounds was unexpected, since other simple aminothiazole and aminopyridine derivatives (2) are relatively powerful inhibitors of the growth of tubercle bacilli. The amebacidal activity of one of the compounds may be due in part to the low degree of water solubility of these triazines.

The Promizole derivative was also found to be a potent carbonic anhydrase inhibitor, a concentration of 48×10^{-7} *M* inhibiting 50% of the carbonic anhydrase activity. In comparison to the activity of Diamox®, this inhibiting amount is in the ratio of 480:1. When possible gastric inhibitor activity on pyloric-ligated rats was determined with this compound, however, no effect was observed after subcutaneous injection on either volume or hydrochloride concentration at levels of 20 or 80 mg./Kg.

Anticancer tests were conducted for several of the triazines, and the results are given in Table III. These tests were carried out against adenocarcinoma 755 in mice, and tumor weight 50% or less of the control was considered significant. Only one of the compounds, 2,4-dichloro-6-(β-diethylaminoethylamino)-s-triazine, may be considered, therefore, to show positive activity.

TABLE I.—*In Vitro* ANTITUBERCULAR ACTIVITIES OF AMINO-S-TRIAZINES[a]

Amino-s-triazine	Concn. mg./ml.	Amount of Growth[b] 11 Days	21 Days
2-Promizolyl-4,6-dichloro	0.02	+++	++++
2,4-bis-(2′-pyridylamino)-6-chloro	0.20	++	++++
2,4,6-tris-(2′-thiazolylamino	0.20	+	+++
2,4,6-tris-(β-diethylaminoethylamino)	0.20	+++	++++
Control		+++	++++

[a] Determined at the Lilly Research Laboratories by C. G. Culbertson.

[b] Modified Proskauer and Beck medium was used with *M. tuberculosis* HRv.

The preparation of another series of amino triazines was attempted by reaction of cyanuric chloride with ethyl carbamate, ethyl glycinate, and ethyl β-alaninate. The products would constitute a homologous series sterically but not chemically, since the amine function of ethyl carbamate is not essentially basic. It was considered that these compounds would provide suitable anticancer candidates, since the ester functions of such amino esters are reactive acylating agents. This provides an analogy to the tumor-inhibiting triazines with

TABLE II.—*In Vivo* ANTITUBERCULAR ACTIVITY OF 2,4,6-TRIS-(β-DIETHYLAMINOETHYLAMINO)-S-TRIAZINE TRIHYDROCHLORIDE[a]

Compound	% in Diet	No. of Mice	Days on Test	Mortality, %	Mean Survival Time in Days
Triazine	0.1	9	19	87	17.1
Triazine	0.02	9	19	66	16.8
PASA	1.0	9	19	11	18.3
Control	...	10	19	90	15.6

[a] Determined at the Lilly Research Laboratories by C. G. Culbertson.

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active alkylating functions, such as triethylenemelamine (TEM).

The synthesis of the glycine and β-alanine derivatives was comparatively simple, since the amine groups were sufficiently basic to be acylated by cyanuric chloride. The reactions were carried out in aqueous media as previously described (1), although a twophase system of chloroform-water was found necessary for the mono-β-alanine derivative, since β-alanine ester was too insoluble in cold aqueous acetone. The 2,4-bis-(β-alanine) derivative could be prepared in the usual manner, however, but the yield was considerably lessened if the reaction was alkaline or a solvent of dioxane-water was used. Although free α-amino acid esters, which were formed *in situ*, readily dimerize on heating, no evidence of diketopiperazine formation was seen in the preparations of the 2,4-bis- and 2,4,6,-tris-(glycine) derivatives. The new triazines prepared are listed in Table IV.

The failure of the third halogen of cyanuric chloride could not be replaced by weak amines, such as nitroanilines, nor the observation of Banks (4) that trisubstituted triazines would not form in alkaline media seemed to be pertinent here, since the amino esters used are fairly strong bases, and the use of neutral media did not favor the condensation. It is possible that the precedence of hydrolysis of the third halogen over condensation with the amines may be due to hydrogen-bonding with water by the two amino nitrogens already attached to the triazine ring. This could facilitate attack by water of the carbon-chlorine bond, a reaction already postulated by Thurston (5) on storage of amino chloro triazines, from aqueous mixtures, containing hydrogen on the amine groups.

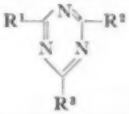
Another route to the formation of the trisubstituted triazines as well as a method for introduction of the urethan group involved the use of an N-sodium derivative of melamine (2,4,6-triamino-s-triazine). This could then be condensed with ethyl chlorocarbonate or other chloro esters. Since Dudley (6) had determined that melamine is a considerably weaker base than ammonia, reaction with sodamide should produce a sodium derivative of melamine. When the reaction was carried out in

TABLE III.—ANTICANCER ACTIVITY OF AMINO-S-TRIAZINES[a]

s-Triazine	Vehicle	Dose, mg./Kg. × No. Times Adm.	Mortality	Av. Mouse Wt. Change, Gm.	Tumor Wt. % of Control
2,4-bis-(2′-Thiazolylamino)-6-chloro	CMC	166 × 6	0/10	+1.1	58
2-(2′-Thiazolylamino)-4,6-dichloro	CMC	63 × 6	1/10	+3.0	72
2-(β-Diethylaminoethylamino)-4,6-dichloro	CMC	10 × 6	0/10	+2.1	43
2,4-bis-(β-Carbethoxyethylamino)-6-chloro	CMC	200 × 6	0/10	+2.3	52
2-(β-Carbethoxyethylamino)-4,6-dichloro	CMC	50 × 6	0/10	+1.0	55
2,4-bis-(Carbethoxymethylamino)-6-chloro	CMC	166 × 6	0/10	+3.7	70

[a] Determined at the Southern Research Institute, Birmingham, Ala. Results were made available by Dr. R. W. Brockman.

TABLE IV.—CARBETHOXYALKYLAMINO-S-TRIAZINES



R^1	R^2	R^3	M. p.[a]	Recrystn. Solvent	Yield, %	Formula	Nitrogen Calcd.	Nitrogen Found[b]
NH-CH_2-CO_2-C_2H_5	Cl	Cl	90–91°	Ethanol	80	$C_7H_9O_2N_4Cl_2$	22.32	22.69
NH-CH_2-CO_2-C_2H_5	NH-CH_2-CO_2-C_2H_5	Cl	179–180°	Butanol	76	$C_{11}H_{14}O_4N_5Cl$	22.04	22.35
NH-CH_2-CO_2-C_2H_5	NH-CH_2-CO_2-C_2H_5	OH	267° (decompn.)			$C_{11}H_{17}O_5N_5$	23.40	23.76
NH-CH_2-CH_2-$CO_2C_2H_5$	Cl	Cl	94–95°		70	$C_8H_{10}O_2N_4Cl_2$	21.14	21.34
NH-CH_2-CH_2-$CO_2C_2H_5$	NH-CH_2-CH_2-$CO_2C_2H_5$	Cl	139–140°	Methanol	85	$C_{13}H_{20}O_4N_5Cl$	20.37	20.32
NH-CH_2-CH_2-$CO_2C_2H_5$	NH-CH_2-CH_2-$CO_2C_2H_5$	OH	273–275° (decompn.)			$C_{13}H_{21}O_5N_5$	21.40	21.71

[a] The melting points are uncorrected.
[b] The nitrogen analyses were obtained by the semimicro Kjeldahl method.

Attempts to prepare the 2,4,6,-trisubstituted triazines in an aqueous medium were unsuccessful, mixtures of the 2,4,-disubstituted-6-chloro and -6-hydroxy derivatives being obtained. Neither the findings of von Meyer (3) that the third halogen of

boiling xylene, ammonia was slowly liberated, but the product gave no evidence of reaction with ethyl chlorocarbonate. Direct acylation of urethan with cyanuric chloride in the absence of solvent at steam-bath temperature, analogously to the acylation of urethan with acetyl chloride or bromide (7), was also unsuccessful in producing a urethan derivative. Products were obtained, however, which gave higher values for nitrogen on analysis than either of the starting substances, and which could be condensation polymers. Condensation of cyanuric chloride and urethan did not take place in refluxing solvents, however.

Biological tests which were carried out on the aminoester derivatives prepared include anticancer determinations, the results of which are shown in Table III. None of the compounds tested gave better than a 50% reduction in tumor weight. Tests for diuretic activity at the Lilly Research Laboratories were negative for the two disubstituted amino esters, 2,4-bis-(carbethoxymethylamino)-6-chloro-s-triazine and 2,4-bis-(β-carbethoxyethylamino)-6-chloro-s-triazine. Oral doses of 10–40 mg./Kg. produced no diuresis in dogs. This result agrees with the finding of Clauder and Bulcsu (8) that bis-amino triazines are most successful as diuretics when no chlorine is attached to the triazine ring. Acute oral toxicity determinations in mice showed that single doses of 1,000 mg./Kg. of these two compounds failed to kill, which indicates a detoxifying effect on cyanuric chloride by the amino acid esters. Cyanuric chloride has an oral LD_{50} of 1,000 mg./Kg. in mice (9). Antibacterial tests were also made with the same two triazines, and of thirty-eight microorganisms observed, only one was inhibited by the ethyl glycinate derivative.

EXPERIMENTAL

Amino Acid Ester Hydrochlorides—The method of Harries and Weiss (10) was used, in which a suspension of the amino acid in absolute ethanol was saturated with dry hydrogen chloride and refluxed until homogeneous. Ethyl glycinate hydrochloride, m. p. 142–144° was prepared in 85% yield, and ethyl β-alaninate hydrochloride, m p. 48–50°, was obtained in 90% yield.

Carbethoxyalkylamino-chloro-s-triazines.—A solution of 4.6 Gm. (0.025 mole) of cyanuric chloride in 40 ml. of warm acetone was poured in a fine stream into a cold (0°) solution of sodium bicarbonate (0.025 mole in 60 ml. of water) with stirring. To the resulting slurry was added 0.025 mole of amino acid ester hydrochloride in small portions along with another equivalent (0.025 mole) of sodium bicarbonate solution during one hour. The reaction mixture was stirred for a period of one-half to two hours, the temperature for a monoamino-dichloro triazine being kept at 0–5°, and for a diamino-monochloro triazine at 45°. The product was collected on a filter, washed free of chloride ion with cold water, and pressed as dry as possible. After drying at 110° for three hours, or overnight *in vacuo*, the product was recrystallized.

In the preparation of 2-(β-carbethoxyethylamino)-4,6-dichloro-s-triazine, it was necessary to use a chloroform solution (40 ml.) of cyanuric chloride. During the reaction, another 60 ml. of chloroform was added, and the product was isolated by separation and evaporation of the chloroform layer. No further purification was necessary.

Attempted preparation of tris-(Aminoester) Triazines: bis-(Carbethoxyalkylamino)-hydroxy-s-triazines.—To a stirred solution of 3.2 Gm. (0.038 mole) of sodium bicarbonate in 50 ml. of water was added a solution of 2.3 Gm. (0.0125 mole) of cyanuric chloride in 25 ml. of dioxane. To this was added 0.0125 mole of amino acid ester hydrochloride, and the solution was refluxed for two hours, with the simultaneous addition of another 3 Gm. of sodium bicarbonate. The product was filtered, washed free of chloride ion with water, and dried at 110° overnight. It was then extracted with acetone in a Soxhlet extractor for several hours, and the acetone-insoluble fraction was saved. The acetone-soluble fraction was found to be identical with the bis - (carbethoxyalkylamino)-chloro-s-triazines already prepared.

SUMMARY

1. The preparation of amino ester derivatives of the s-triazine ring is described. Glycine and β-alanine esters were condensed successfully with cyanuric chloride, but urethan gave no characterizable product.

2. The results of a number of biological tests of these and other triazines are listed. Antitubercular, amebacidal, anticancer, and carbonic anhydrase inhibiting activities were observed.

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Chelating Agents as Color Stabilizers for Epinephrine Hydrochloride Solutions*

By CHARLES W. ROSCOE and NATHAN A. HALL

Some amino acid derivatives have been examined for their effect on inhibition of discoloration of epinephrine hydrochloride solutions buffered at *p*H 4.0. The chelating agents were studied alone and in combination with sodium bisulfite. The efficacy of these agents was evaluated by comparing their ability to retard the discoloration of epinephrine hydrochloride solutions in the presence and absence of ferric iron. The progress of color formation was followed spectrophotometrically for periods up to forty-five days. In the absence of iron contaminant, the chelating agents were found to be of little value in preventing discoloration. In the presence of iron contaminant, Sequestrene® NA2, Versenol®, and Perma Kleer®-80, in that order, significantly inhibited color formation and their efficacy was enhanced with the addition of sodium bisulfite. Versene® Fe-3 Specific was of little value under these conditions.

THE INSTABILITY of aqueous epinephrine solutions has long been a matter of interest and concern to the pharmacist. It is well known that adrenaline in aqueous solution readily undergoes oxidation which is accelerated by light, heat, oxygen, an increase in *p*H, and by the presence of certain metallic ions. The reaction is further autocatalytically stimulated by the presence of a red-colored quinonoid compound called adrenochrome, which is formed during the initial stages of oxidation and which imparts a red color to the oxidizing epinephrine solution.

The catalytic effect of various metallic ions, particularly iron and copper, on the oxidation of aqueous epinephrine solutions has been the subject of numerous reports. Chaix, *et al.* (1), believed the uptake of oxygen in their epinephrine control solutions to have been due to traces of metallic impurities present in the apparatus or reagents. Gelebart (2), studying the stability of procaine solutions containing epinephrine, found that procaine acted as a catalyst in the oxidation of epinephrine. This was attributed to the presence of impurities in the procaine. Girard and Kerny (3) reported that certain ampuls made of yellow glass contained sufficient iron to interfere with the preservation of aqueous epinephrine hydrochloride solutions.

Various antioxidants such as the sulfites and ascorbic acid have been used as epinephrine stabilizers with varying degrees of success. West, *et al.* (4–8), have given considerable attention to the use of sodium metabisulfite in epinephrine hydrochloride solutions. Amino acids such as glutathione, cysteine, and alanine (9–11) have been reported to be effective in retarding the oxidation of epinephrine solutions containing certain metallic ions.

Recently the introduction of ethylenediaminetetraacetic acid (EDTA) and its derivatives has created new interest in problems involving the control of metallic ions in solution. These amino acid derivatives, by virtue of their strong metallic ion chelating properties, have enjoyed wide application in the fields of chemistry, biology, and medicine. In the pharmaceutical field, EDTA compounds have been used to inhibit the copper-catalyzed oxidation of ascorbic acid in solution (12, 13). Their application to the stabilization of a Terramycin® solution (14) and some penicillin solutions (15) has also been reported. Crismon (16) observed that the intravenous injection of disodium EDTA into rats markedly increased the epinephrine sensitivity of the precapillary sphincters in the mesoappendix. When sufficient iron as ferrous sulfate was added to the EDTA salt to form the iron chelate, neither the marked depression of epinephrine sensitivity produced by iron nor the marked sensitization usually seen with the iron-free EDTA occurred.

While work on the present paper was in progress, a preliminary report by Ruggiero and Blake (17) on the use of EDTA salts as color stabilizers for epinephrine was brought to our attention. They reported that disodium EDTA in 0.05% concentration significantly inhibited color formation in 0.1 to 0.2% epinephrine solutions. When reduced iron was added as a contaminant, color formation was retarded by both disodium EDTA and disodium calcium EDTA.

On the basis that the degree of color formation in epinephrine solutions provides an index to the

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extent of oxidation taking place, the authors have undertaken a spectrophotometric study to evaluate an EDTA derivative and several other amino acid derivatives as substances to prevent discoloration in epinephrine hydrochloride solutions. Some preliminary experiments were first necessary to determine the most suitable conditions for the study. Such factors as the wavelength to be used for measuring absorbance; the metal contaminant and its concentration; and *p*H were considered.

EXPERIMENTAL

Materials.—(*a*) Sodium bisulfite stock solution 10% was freshly prepared for each experiment. (*b*) Ascorbic acid stock solution 10% was freshly prepared for each experiment. (*c*) Ferric chloride stock solution (containing ferric ion at concentration 1:10,000) was freshly prepared for each experiment by dissolving 0.122 Gm. of $FeCl_3 \cdot 6H_2O$ in 250 ml. distilled water. (*d*) Levo-epinephrine (synthetic) as purchased from the Eastman Kodak Co. (white label) was stored in the dark, at room temperature, and in a soda lime desiccator. (*e*) Clark-Lubs phthalate buffer solution, *p*H 4.0, was freshly prepared for each experiment as a 0.4 *M* stock solution. (*f*) Chlorobutanol preservative stock solution 0.8%. (*g*) Distilled water used in preparing all solutions was redistilled from Pyrex® glass. (*h*) Chelating agents and concentrations used in this study are listed in Table I.

TABLE I.—CHELATING AGENTS

Compound	Composition	Concentration, %[a]
Sequestrene NA2[b]	Disodium ethylenediaminetetraacetate dihydrate	0.7
Perma Kleer-80[c]	Amino-carboxylate type compound[e]	0.03
Versenol[d]	Trisodium N-hydroxyethylethylenediaminetriacetate	0.06
Versene Fe-3 Specific[d]	Sodium N, N-dihydroxyethyl glycine	0.02

[a] Based upon technical information supplied by the manufacturer. The concentrations represent ten times the quantity calculated to chelate 10 mg. of ferric ion.
[b] Supplied through the courtesy of the Alrose Chemical Co., Providence 1, Rhode Island.
[c] Supplied through the courtesy of the Refined Products Corp., Lyndhurst, N. J.
[d] Supplied through the courtesy of the Bersworth Chemical Co., Framingham, Mass.
[e] No further information on this compound was supplied by the manufacturer.

Selection of Wavelength for Spectrophotometric Study.—An examination of the ultraviolet absorption spectra of aqueous epinephrine hydrochloride solutions, using 1-cm. silica cells in a Beckman quartz spectrophotometer (Model DU), revealed the presence of absorption maxima at wavelengths of 220–224 mμ and 280 mμ. From the spectrograms in Fig. 1, it can be seen that there was no significant difference between the spectral curve of a 1:10,000 solution of epinephrine hydrochloride which had been stored for three days and one which had been stored for seventeen days at room temperature.

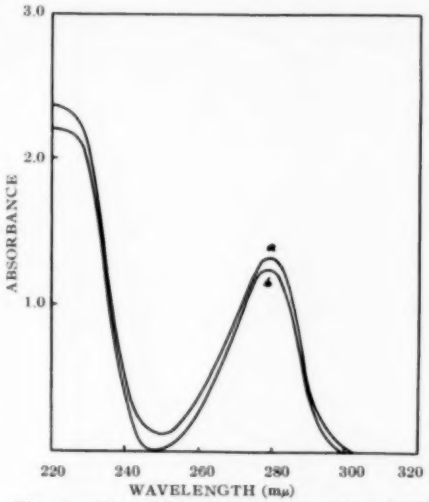

Fig. 1.—Ultraviolet absorption spectra of epinephrine hydrochloride solutions 1:10,000. (*a*) 1:10 dilution of 1:1000 epinephrine solution after storage for 3 days at room temperature; initial *p*H 4.0; solution colorless. (*b*) 1:10 dilution of 1:1000 epinephrine solution after storage for 17 days at room temperature; initial *p*H 4.0; solution light red.

However, the spectral curves of old discolored epinephrine hydrochloride solutions at a concentration of 1:1000 showed that significant changes in absorbance had taken place, particularly in the region beginning at a wavelength of 300 mμ and extending into the visual spectrum (Fig. 2).

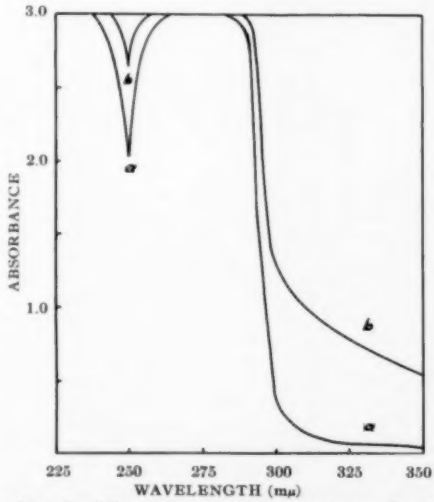

Fig. 2.—Ultraviolet absorption spectra of epinephrine hydrochloride solutions 1:1000. (*a*) Stored for 5 days at room temperature; initial *p*H 4.0; solution pink. (*b*) Autoclaved for thirty minutes at 15 pounds, then stored at room temperature for 30 days; initial *p*H 4.0; solution brown.

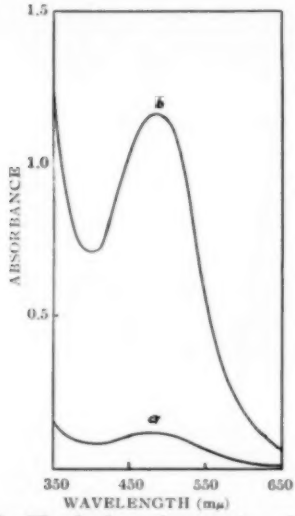

Fig. 3.—Visual absorption spectra of 1:1000 epinephrine hydrochloride solutions containing iron catalyst. Spectra taken fifteen minutes after preparation of samples. (*a*) 10 p. p. m. ferric ion added; initial *p*H 4.0; solution pink. (*b*) 100 p. p. m. ferric ion added; initial *p*H 4.0; solution deep red.

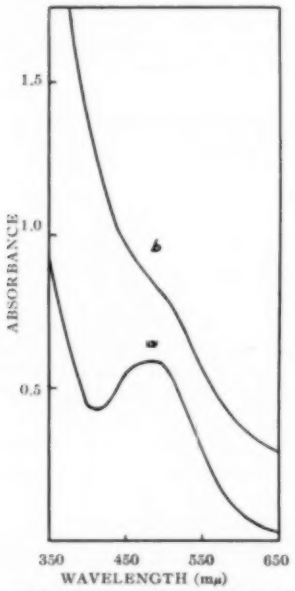

Fig. 4.—Visual absorption spectra of 1:1000 epinephrine hydrochloride solutions containing iron catalyst. Spectra taken ninety-six hours after preparation of samples. (*a*) 10 p. p. m. of ferric ion added; initial *p*H 4.0; solution light red. (*b*) 100 p. p. m. of ferric ion added; initial *p*H 4.0; solution reddish-brown with precipitate.

The spectrograms in Fig. 3 and 4 revealed the changes in the visual spectra of epinephrine hydrochloride solutions in the presence of ferric ions. The absorption peaks in the region 480–490 mμ for the pink to red solutions probably belong to adrenochrome. An absorption maximum for adrenochrome in the visual spectrum has been reported to range from 365–540 mμ (10, 18, 19). At first glance, it appeared that a wavelength in the vicinity of 480–490 mμ might be suitable for measuring the the absorbance of oxidizing epinephrine solutions. However, since the formation of adrenochrome represents only the initial stages of epinephrine oxidation, the authors believed that readings taken in this region of the spectrum would give an incomplete picture of the reaction. Additional studies indicated that the spectral curves of oxidizing epinephrine solutions containing inhibitors did not always show the characteristic absorption peak for adrenochrome, which suggested the possibility of a different oxidative pathway.

Further examination of the spectrograms revealed that the use of a wavelength of 350 mμ might be more suitable for following the progress of epinephrine oxidation over an extended period of time. The spectral curves in Fig. 3 and 4 illustrated how absorbance taken at 350 mμ permitted the use of a major portion of the spectrophotometer density scale as the epinephrine solutions progressed from a pink to reddish-brown color with subsequent precipitation.

Selection of a Metal Catalyst.—In a preliminary study, the following metallic ions were examined for their effects on the acceleration of color formation in epinephrine hydrochloride solutions: Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Cr^{3+}, Pb^{2+}, Sn^{4+}, Sn^{2+}, Al^{3+}, Hg^{2+}, Ni^{2+}, and Mn^{2+}. A solution of the metal salt was added to the appropriate epinephrine solution, previously adjusted to *p*H 4.0, to give concentrations of the metallic ion of 1 p.p.m., 10 p.p.m., and 100 p.p.m. The resulting solutions were stored in 10-ml. glass-stoppered flasks, and observed for color formation over a period of two weeks at room temperature.

In the solutions containing 1 p.p.m. of metallic ion, only ferric and ferrous ions produced a coloration slightly greater than that found in the epinephrine control solution, which was a moderate pink color after two weeks. Ferric, ferrous, cupric, and chromic ions at concentrations of 10 p.p.m. and 100 p.p.m. produced deep reddish-brown solutions or suspensions. Of the remaining metallic ions, only manganese and aluminum at 100 p.p.m. produced a coloration greater than that found in the epinephrine control. As a result of this study, the ferric ion was chosen as the catalyst to be used in subsequent experiments.

The spectral curves in Fig. 3 and 4 illustrate more clearly the accelerating effect of the ferric ion on the rate of color formation in oxidizing epinephrine solutions. A concentration of 10 p.p.m. of ferric ion was selected as being most satisfactory for this investigation.

Preliminary *p*H Studies.—That the color stability of aqueous epinephrine solutions decreases as the *p*H increases was reaffirmed. Preliminary experiments with unbuffered epinephrine solutions also showed that there was a tendency for the *p*H to

decrease as oxidation progressed, with subsequent adverse effects on the course of color formation. Obviously, it would be difficult to evaluate the stabilizers under these conditions. In order to maintain a constant *p*H, a Clark-Lubs phthalate buffer (*p*H 4.0) was added to all epinephrine solutions. Routine determinations of *p*H on the buffered solutions at various stages of oxidation showed that the phthalate buffer was adequate.

Comparative Effects of Chelating Agents in Buffered Epinephrine Hydrochloride Solutions.—The chelating agents under study were evaluated as color stabilizers in epinephrine hydrochloride solutions buffered at *p*H 4.0. The efficacy of these agents was measured by their ability to inhibit the discoloration of epinephrine solutions in the presence and absence of added ferric ions. The progress of color formation was followed by taking absorbance at a wavelength of 350 mμ for periods up to forty-five days, using a Beckman quartz spectrophotometer (Model DU) with a tungsten lamp. For each reading, the instrument was first balanced against a corresponding blank which was identical to the parent solution except for the absence of epinephrine. The results were recorded by plotting the absorbance (at 350 mμ) against time (in days). The inhibiting effect of sodium bisulfite, used alone and in combination with ascorbic acid, served as basis for comparison. The chelating agents were studied in four series of epinephrine solutions as follows:

Series I—Buffered epinephrine hydrochloride solutions containing a chelating agent.

Series II—Buffered epinephrine hydrochloride solutions containing a chelating agent in the presence of added iron.

Series III—Buffered epinephrine hydrochloride solutions containing a chelating agent in combination with bisulfite.

Series IV—Buffered epinephrine hydrochloride solutions containing a chelating agent in combination with bisulfite and in the presence of added iron.

Procedure.—One hundred milliliters of a 1:500 solution of epinephrine hydrochloride was freshly prepared for each series by dissolving 200 mg. of *levo*-epinephrine in 10 ml. of 0.1 *N* HCl and by adding sufficient chlorobutanol stock solution to make a final volume of 100 ml. Five-milliliter portions of this solution were pipetted into all flasks which were to contain epinephrine. Four and one-half milliliters of the chlorobutanol solution was pipetted into all flasks which were to contain blanks. The color stabilizers were next added at their appropriate concentrations. The solutions were then adjusted to *p*H 4.0 with 0.1 *N* HCl. Two and one-half milliliters of the 0.4 *M* buffer solution was added to all solutions to give a final buffer concentration of 0.1 *M*. To those solutions which were to contain iron, 1 ml. of the 1:10,000 solution of ferric ions was added to give a final concentration of 10 p.p.m. The solutions were finally made up to 10 ml. with distilled water, and the absorbances at 350 mμ were read immediately and thereafter at appropriate intervals.

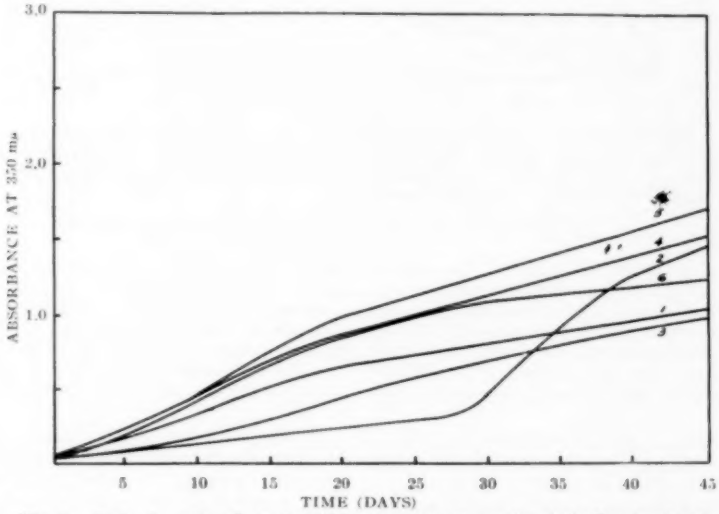

Fig. 5.—Series I. The effect of stabilizers on color formation in buffered epinephrine hydrochloride solutions. (1) epinephrine control; (2) bisulfite; (3) Sequestrene NA2; (4) Perma Kleer-80; (5) Versenol; (6) Versene Fe-3 Specific.

RESULTS

Series I.—The results of this series are summarized in Fig. 5. Color formation in the epinephrine solution containing bisulfite (Solution 2) was re-

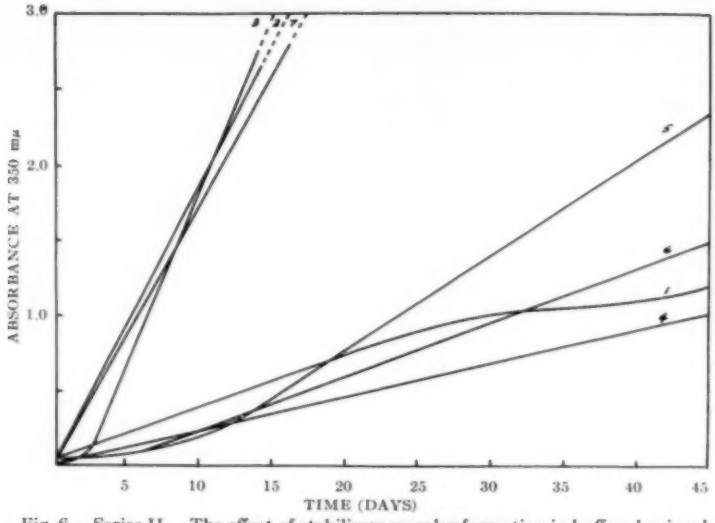

Fig. 6.—Series II. The effect of stabilizers on color formation in buffered epinephrine hydrochloride solutions containing iron contaminant: (1) epinephrine control; (2) epinephrine plus iron; (3) bisulfite; (4) Sequestrene NA2; (5) Perma Kleer-80; (6) Versenol; (7) Versene Fe-3 Specific.

tarded for about twenty-eight days. Thereafter, a sharp increase in the rate of color formation was observed. In Solution 3, color formation appeared to be slightly retarded by Sequestrene NA2, but the remaining chelating agents were of no value.

The color of these solutions ranged from a trace of pink after forty-eight hours to a brownish-orange after forty-five days. Solution 3 did not show any color until after eight days. Solution 2 remained colorless for two weeks.

A solution containing ascorbic acid was prepared for this series, but after four days the blank developed a yellow color which gave an absorbance almost as great as that of the parent solution. For

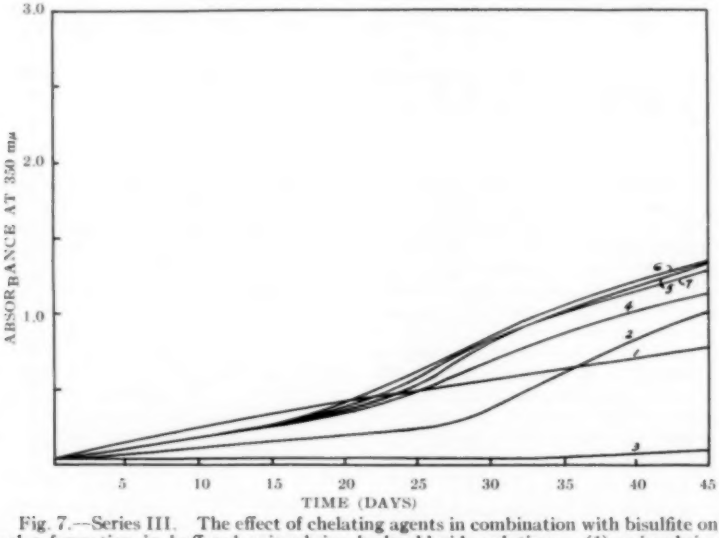

Fig. 7.—Series III. The effect of chelating agents in combination with bisulfite on color formation in buffered epinephrine hydrochloride solutions: (1) epinephrine control; (2) bisulfite; (3) bisulfite plus ascorbic acid; (4) bisulfite plus Sequestrene NA2; (5) bisulfite plus Perma Kleer-80; (6) bisulfite plus Versenol; (7) bisulfite plus Versene Fe-3 Specific.

this reason, the ascorbic acid solution was omitted from the series. Blanks for the other solutions remained colorless throughout the study.

Series II.—The results of this series are summarized in Fig. 6. Bisulfite (Solution 3) and ranged from a trace of yellow to light orange. These solutions were also free from sediments.

Series IV.—The results of this series are summarized in Fig. 8. With the exception of Versene Fe-3 Specific, all of the chelating agents in com-

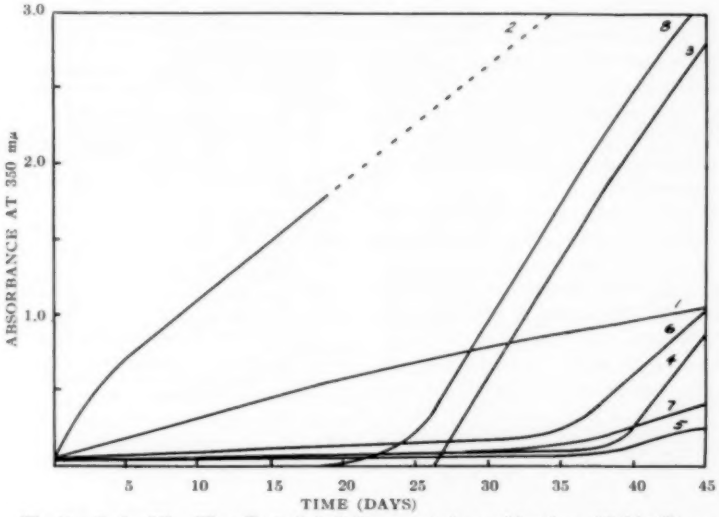

Fig. 8.—Series IV. The effect of chelating agents in combination with bisulfite on color formation in buffered epinephrine hydrochloride solutions containing iron contaminant: (1) epinephrine control; (2) epinephrine plus iron; (3) bisulfite; (4) bisulfite plus ascorbic acid; (5) bisulfite plus Sequestrene NA2; (6) bisulfite plus Perma Kleer-80; (7) bisulfite plus Versenol; (8) bisulfite plus Versene Fe-3 Specific.

Versene Fe-3 Specific (Solution 7) were ineffective as color stabilizers in the presence of iron. The remaining stabilizers, however, definitely inhibited the catalytic effect of iron. Of these, Sequestrene NA2 (Solution 4) was the most effective.

All solutions in this series developed a trace of color with the addition of iron. Solutions 2, 3, and 7 were dark brown after eight days and contained precipitates after two weeks. After forty-five days, the remaining solutions varied in color from light orange (Solution 4) to brownish-orange (Solution 5). The latter solutions contained small amounts of sediment.

Series III.—The results of this series are summarized in Fig. 7. The chelating agents in combination with bisulfite (Solutions 4, 5, 6, and 7) were ineffective as color stabilizers in this series. The results were comparable to those obtained in Series I, except in the case of Sequestrene NA2 which appeared to be less effective in the presence of bisulfite.

As in Series I, bisulfite alone (Solution 2) offered some protection initially, but later was of no value. Bisulfite in combination with ascorbic acid (Solution 3), however, markedly inhibited color formation. Only after thirty-five days was there any significant increase in the absorbance of this solution. The efficacy of bisulfite appeared to be greatly enhanced by the addition of ascorbic acid.

All solutions containing bisulfite remained colorless for two weeks. After forty-five days, the colors

bination with bisulfite afforded good protection against the catalytic effect of iron for thirty-five days or more. It was apparent that the efficacy of the chelating agents in the presence of iron was enhanced by the addition of bisulfite. Sequestrene NA2 again appeared to be the most effective of these compounds.

Solutions 4, 5, 6, and 7 remained nearly colorless for about thirty-five days. After forty-five days, the colors varied from a trace of orange to brown. All solutions containing bisulfite were free from sediments.

Reports which attempt to correlate color formation in epinephrine solutions with physiological activity are not in good agreement (4, 10, 20). Nevertheless, it is known that color formation in these solutions indicates oxidation of the parent compound which eventually gives rise to precipitation and loss of physiological activity. In order to completely evaluate the effectiveness of the chelating agents as stabilizers, it appears that further studies should be undertaken, including their effect upon racemization and physiologic activity.

SUMMARY

1. A spectrophotometric procedure was devised for following the progress of color formation in oxidizing epinephrine hydrochloride solutions.

2. A number of metallic ions were briefly examined for their catalytic effect on the rate of color formation in epinephrine hydrochloride solutions. Ferric, ferrous, cupric, and chromic ions were found to be the most active. The ferric ion was selected for the purpose of this study.

3. In the absence of iron contaminant, the chelating agents were of limited value in retarding color formation in the epinephrine solutions under study. The addition of bisulfite produced no better results. Bisulfite in combination with ascorbic acid, however, proved to be effective under these conditions.

4. In the presence of iron contaminant, the chelating agents inhibited color formation to varying degrees. At the concentrations used, these compounds were effective in the order: Sequestrene NA2> Versenol> Perma Kleer-80> Versene Fe-3 Specific. The efficacy of the chelating agents was considerably enhanced in the presence of bisulfite. When used alone bisulfite was of limited value under these conditions, but the addition of ascorbic acid greatly increased its effectiveness.

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Prediction of Stability in Pharmaceutical Preparations III. Comparison of Vitamin Stabilities in Different Multivitamin Preparations*

By EDWARD R. GARRETT

Further proof of the validity of a predictive method for room temperature stability is given and possible limitations are discussed. Certain data may indicate similarity of degradation mechanism and heats of activation and thus allow minimum effort in prediction of stability of slightly modified preparations or of preparations to be kept within a manufacturer's control limits.

PRIOR PUBLICATIONS in this series (1, 2) have outlined and applied physicochemical and statistical procedures for the prediction of room temperature stability of pharmaceutical preparations.

The effect of changing vehicles and compositions of pharmaceutical preparations on the parameters of the predictive equations is of practical importance in that it may determine the need, or lack of need of complete studies of this type. This study compares the prediction of stability in different liquid multivitamin preparations and provides further proof of the effectiveness of the methods.

* Received January 20, 1956, from the Research Division, The Upjohn Company, Kalamazoo, Mich.

EXPERIMENTAL[1]

The thermal degradations of components of a multivitamin preparation were studied by assaying the components after various times of maintenance at elevated temperatures. Fifty cubic centimeter aliquots of a preparation were placed in 60-cc. amber, square, screw-cap bottles with vinylite-lined caps and incubated at 70, 60, 50, 40, and 4°. The procedures of preparation, sampling, and assay of the multivitamin preparation were the same as in the prior publication in this series (2). The components studied were vitamin B_{12}, *d*-pantothenyl alcohol and thiamine hydrochloride.

[1] The cooperation of Dr. David H. Gregg of the Pharmaceutical Development Department of The Upjohn Company for formulation of the preparations and submission of samples for assay is gratefully acknowledged. Grateful recognition is also given to the Control Division of The Upjohn Company for the vitamin assays.

The product, (A) was prepared so that each 15 cc. theoretically contained 43.17 mg. ferrous gluconate powder N. F., 100 mg. calcium glycerophosphate N. F., 5.45 mg. thiamine hydrochloride U. S. P., 3.47 mg. riboflavin U. S. P., 1.2 mg. vitamin B_6 hydrochloride, 35.0 mg. nicotinamide U. S. P., 3.96 mg. *d*-pantothenyl alcohol, 4.95 μg. vitamin B_{12}, 56.62 mg. tricholine citrate, 0.033 mg. cobalt (as sulfate), 0.33 mg. copper (as sulfate), 0.33 mg. manganese (as sulfate), 2.0 mg. magnesium (as sulfate), 0.066 mg. molybdenum (as sodium molybdate), 0.50 mg. zinc (as sulfate), and flavoring agents. The vehicle consisted of 60% sugar, granulated, U. S. P., and 19% alcohol with tragacanth powder U. S. P., as a thickening agent. The *p*H was adjusted to 3.7 with hydrochloric acid and no change in *p*H was noted throughout the study. Standard procedures were used in the assay of thiamine hydrochloride, *d*-pantothenyl alcohol, and vitamin B_{12} in this preparation.

Another lot, (A′) of the same composition as A, was prepared and also studied for room temperature shelf life stability to test the predictions based on preparation A's accelerated temperature studies.

The methods of data treatment and calculation in this paper are the same as in the prior papers of this series (1, 2). The figures used as examples were chosen for the vitamins whose rate data were not previously presented in such detail.

Figure 1 is an example of the pseudo first order[2]

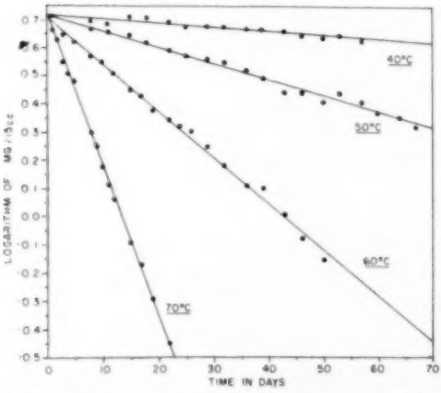

Fig. 1.—First order plots of the thermal degradation of thiamine hydrochloride in a liquid multivitamin preparation (A); logarithms of concentration (mg./15 cc.) against time in days.

thermal degradations at 70, 60, 50, and 40° of a vitamin component in A, thiamine hydrochloride. The rate constants, k and their standard deviation (σ_k) as determined from least squares regression analysis on assay values (or log assay values) against time for n data are tabulated in Table I. This table also includes the values assigned to the parameters of the Arrhenius expression $\log k = P - S/T$; $S = \Delta H_a/2.303\,R$ obtained from the least squares regression analysis of the logarithm of the rate constant ($\log k$) against the reciprocal of the absolute temperature ($1/T$). The symbol σ_s represents the standard error in the Arrhenius slope; $\sigma_{\log k}$, the standard deviation ascribed to $\log k$; and N, the number of temperatures at which degradation was studied.

[2] In general, zero or first order treatment of the rate data will serve as adequate representation of assay vs. time. This does not necessarily mean that fractional orders are not possible or actual.

TABLE I.—TABULATION OF SLOPES (k) OF RATE PLOTS, FACTORS OF ARRHENIUS' EQUATION AND THEIR RESPECTIVE STANDARD DEVIATIONS (σ) FOR THE THERMAL DEGRADATIONS OF VITAMINS[a]

Component[b]: Order of Degradation: Units of k		Vitamin B_{12} Zero (k_0) μg./15 cc./day	*d*-Pantothenyl Alcohol Zero (k_0) mg./15 cc./day	Thiamine Hydrochloride First (k_1) (2.303 day)$^{-1}$
70°	k	0.109	0.0919	0.0546
	σ_k	0.009	0.0038	0.0007
	n	24	34	15
60°	k	0.034	0.0357	0.0165
	σ_k	0.003	0.0020	0.0004
	n	20	40	19
50°	k	0.011	0.0149	0.00574
	σ_k	0.003	0.0015	0.00016
	n	23	42	20
40°	k	...	0.0053	0.00138
	σ_k	...	0.0016	0.00048
	n	...	42	7
	P	15.25	11.77	15.15
	S	5,567	4,394	5,631
ΔH_a (Kcal./mole)		25.5	20.1	25.8
	N	3	4	4
	σ_s	432	76	173
10^3	$\sigma_{\log k}$	17.5	15.9	36.0

[a] For definition of symbols, see reference (2).

[b] The standard deviation (of assay) about regression as determined from the least squares analysis was the same as for the assay in the previous paper of this series (2). On the same concentration basis, these standard deviations of assay (σ_y) are for B_{12}, 0.1 μg./5 cc., *d*-pantothenyl alcohol, 0.06 mg./5 cc., and for thiamine hydrochloride, $\sigma_{\log y} = 0.0180$.

Predicted rates and times of maintenance of desired minimum values for assumed initial assay values of the components of the preparations are given in Table II for 30 and 25°. In addition, the estimated standard error of the predicted rates and times of maintenance as derived from fitting the Arrhenius relation are given. Typical plots are shown in Fig. 2 and 3 where the standard error in a predicted rate is shown by the height of the rectangles at 30, 25, and 20°. The vertical lines show the extent of the 95% confidence limits of such predicted rates as based on four temperature studies.

The predicted rate constants are substituted into appropriate zero or first order equations. For a given initial assay value (y_0) and a specified minimum assay value ($y = y_m$) to be maintained, the time of maintenance ($t = t_m$) of this minimum assay value can be computed as in Table II.

The validity of these predictions of stability in such pharmaceutical preparations can be shown for all three vitamins as in the typical Fig. 4 for thiamine hydrochloride, a plot of the assays against time of preparations kept at room temperature. The solid line represents the predicted thermal degradation at the given temperatures. The dashed lines encompass the standard error in the predicted thermal degradations and 95% confidence limits of a single assay.

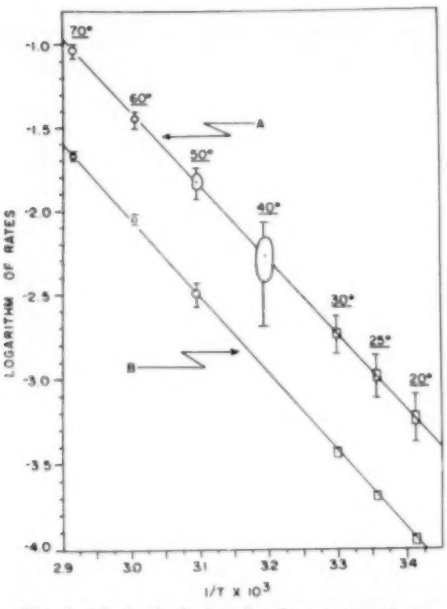

Fig. 2.—Arrhenius' plots for *d*-pantothenyl alcohol. Curve *A* is the plot of the logarithm of the slopes (mg./15 cc./day) of zero order plots against the reciprocal of the absolute temperature (T) for the thermal degradation of *d*-pantothenyl alcohol in liquid multivitamin preparation A. Curve *B* is a similar plot for liquid multivitamin preparation B of the logarithm of the slopes of first order plots (in days). The circles represent the standard deviation of an experimental log rate and the rectangles the standard deviation of a predicted log rate. The horizontal lines represent the 95% confidence limits of experimental and predicted log rates.

DISCUSSION

If a one year maintenance at room temperature of the stated minimum assay values in Table II is desired, the initial amounts of B_{12}, *d*-pantothenyl alcohol and thiamine hydrochloride in preparation A are sufficient.

As had been stated previously (2), the use of a minimum of four temperature studies permits the imposing of finite 95% confidence limits on rate constants predicted from the Arrhenius equation. The extent of the vertical lines in Fig. 2 and 3 for the 30, 25, and 20° values represent these estimated 95% confidence limits.

The vehicle for the preparation A reported in this paper differed from the preparation reported on previously (2), herewith called B. Although the degradation of *d*-pantothenyl alcohol in A is fitted by pseudo zero order and in B by first order kinetics, the slopes of their Arrhenius plots and thus their heats of activation are the same. See Fig. 2 for the parallelism of the Arrhenius plot of A and B. This suggests, but does not necessarily imply, similar mechanisms of degradation.

Similar parallelism of Arrhenius plots of the pseudo first order rate constants of thiamine hydrochloride for both A and B, and thus equivalent heats of activation, are shown in Fig. 3. The fact that the two lines are not superimposable, however, graphically demonstrates that the actual rates at a given temperature differ. This suggests similar mechanisms of the degradation in the vehicles of A and B. Acceptance of this suggestion also implies that possible catalysts affecting degradation are more evident in A.

The heats of activation for degradation of vitamin B_{12} in A and B are also similar within the errors of their estimation.

Although these comparisons for these particular vehicles and vitamin components may suggest similar mechanisms of degradation, it should not be implied that this is true of all vitamin preparations (2). However, it does indicate that if, for a given vehicle, the heat of activation is determined for a particular component it most probably is valid for *slight* modifications of the vehicle and *reasonable* changes in concentration of the component. Thus, a previously determined heat of activation and one rate study at an elevated temperature may satisfactorily predict stability of a component in a modified preparation. The effectiveness of a "flush" or the stability of the same component from a different source may be checked by a short term high temperature study in the same vehicle. This

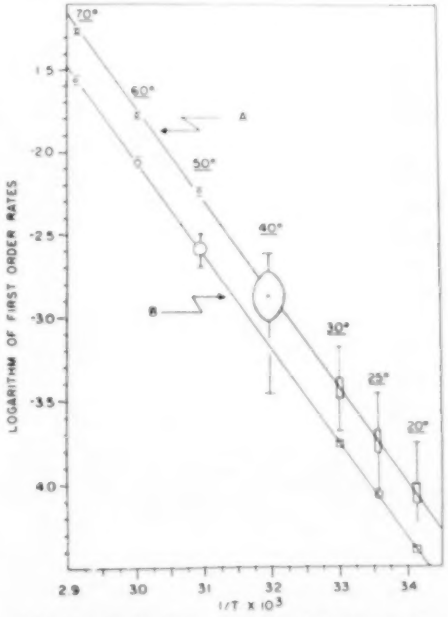

Fig. 3.—Arrhenius' plots for thiamine hydrochloride. Plot of the logarithm of the slopes of first order plots (in days) against the reciprocal of the absolute temperature (T) for the thermal degradation of thiamine hydrochloride in liquid multivitamin preparations A and B. The circles represent the standard deviation of an experimental log rate and the rectangles the standard deviation of a predicted log rate. The horizontal lines represent the 95% confidence limits of experimental and predicted log rates.

TABLE II.—TABULATION OF PREDICTED RATES AND TIMES OF MAINTENANCE OF A STATED MINIMUM ASSAY

Component:	Vitamin B_{12}		*d*-Pantothenyl Alcohol		Thiamine Hydrochloride	
Temperature	30°	25°	30°	25°	30°	25°
Predicted rate constant (k)	7.8×10^{-4}	3.8×10^{-4} μg./15 cc.	1.9×10^{-3}	1.07×10^{-3} mg./15 cc.	3.7×10^{-4}	1.79×10^{-4} (2.303 days)$^{-1}$
Range of std. error of k	(6.9–8.6) $\times 10^{-4}$	(3.2–4.4) $\times 10^{-4}$	1.8–2.0 $\times 10^{-3}$	(0.99–1.15) $\times 10^{-3}$	(2.35–4.25) $\times 10^{-4}$	(1.54–3.08) $\times 10^{-4}$
Years (t_{90}) of maintenance of minimum assay and std. error	5.8 ± 0.7	12 ± 2	0.95 ± 0.05	1.7 ± 0.1	1.5 ± 0.3	3.0 ± 0.5
Minimum assay to be maintained	3.30 μg./15 cc.		3.30 mg./15 cc.		3.30 mg./15 cc.	
Assumed initial assay	4.95 μg./15 cc.		3.96 mg./15 cc.		5.2 mg./15 cc.	

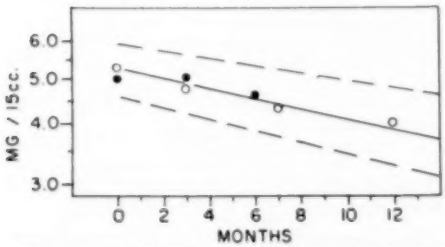

Fig. 4.—Verification of prediction of thiamine hydrochloride stability at room temperature. The solid line is the predicted thermal degradation rate of thiamine hydrochloride in liquid multivitamin preparation A at 30°. The dashed lines encompass standard error in predicted values and the standard error of a single assay. The open circles are actual data obtained on preparation A and the closed circles are actual data obtained on a similar preparation A′.

would result in the practical advantages of even greater economic savings of time and effort once the thermodynamic characteristics of a particular system have been determined. The fact would be of great importance in the normal chemical control process of manufacture.

The use of these physicochemical procedures for prediction of stability must be considered in the light of the controlling mechanisms of degradation. The components of preparation in these papers most probably degrade by solvolytic processes, i. e., reactions in solution, and their heats of activation are generally in the range 10–30 Kcal./mole. Thus, advantage may be taken of significant increases in rate with temperature. However, if diffusion or photolysis are the rate determining steps, the heat of activation is only of the magnitude of 2–3 Kcal./mole and little advantage is gained by accelerated temperature studies in prediction since the temperature effect on rate is small. In some cases, such rates may be accelerated by increases in pressure or light intensities.

Frequently, the thermal degradation of polyhydroxylic materials and the subsequent effect on other components are of interest. However, it must be realized that the heats of activation for such pyrolysis are frequently of the magnitude of 50–70 Kcal./mole. Thus, rates of degradations which may be great at elevated temperatures may not be of any practical significance at the temperature of marketing and storaging of the preparation.

SUMMARY

1. The rates of thermal degradation at elevated temperatures and heats of activation of vitamin B_{12}, *d*-pantothenyl alcohol, and thiamine hydrochloride in a new liquid vitamin preparation have been determined. Predictions of room temperature stability have been confirmed.

2. Slight modifications of vehicle and composition did not significantly change the heats of activation or the rate of change of degradation rate with temperature so that, in many such cases, it may be practical to predict on the basis of one elevated temperature with sufficient confidence. This may serve as an efficient control procedure.

3. The advantages of using accelerated temperatures in a predictive method are limited to degradative mechanisms that have heats of activation of sufficiently high magnitude (i. e., in excess of 10 Kcal./mole). However, too high a heat of activation of components (sugar, etc.) that trigger the degradation of others may give unwarranted estimates of instability.

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Evaluation of Collyria by the Rabbit Eye Method*

By JAMES R. McCOWAN† and WILLIAM J. HUSA

It was found that isotonic solutions of zinc sulfate were significantly less irritating to the rabbit eye than hypotonic solutions. Osmotic pressure was more important than *p*H in reducing the irritation. Zinc sulfate solutions stabilized against precipitation by addition of sodium citrate or potassium sodium tartrate were more irritating than nonstabilized zinc sulfate solutions at lower zinc concentrations but were slightly less irritating in case of solutions of higher zinc concentration. Instillations of zinc sulfate collyria were found to be of no value in reducing the time required for recovery of the rabbit eye from irritation induced by either mustard oil or ultraviolet radiation.

ZINC SULFATE is a constituent of many eye drops and collyria to impart astringency to these preparations for the relief of slight inflammations and hyperemia of the eye. Because of the instability of zinc sulfate solutions near the normal *p*H of the tear fluids and because of their irritating nature, various buffer systems and stabilizers have been used to increase the stability of zinc sulfate solutions at a higher *p*H and to decrease the irritation produced by such preparations.

Many collyria have been formulated on a basis of chemical and physical properties, but very few reports have been made in the literature concerning the actual therapeutic value of such collyria.

Husa and Dale (1) have reported the formulation of collyria containing soluble zinc compounds and conforming to the desired osmotic pressure and *p*H, these collyria being stable at the *p*H of the normal tear fluid. A barbital-sodium barbital buffer with a maximum buffer capacity at the approximate *p*H of the tear fluid was used to maintain the desired *p*H.

The purpose of this investigation was to determine whether the addition of stabilizing agents such as sodium citrate and aminoacetic acid, and the use of a buffered vehicle have any appreciable effect on the irritation produced by zinc sulfate in the irritated rabbit eye, and whether the therapeutic properties of zinc sulfate in the irritated rabbit eye are increased or decreased by the addition of stabilizing agents and use of a buffered vehicle.

EXPERIMENTAL

Preparation of Solutions.—Hypotonic solutions were prepared by dissolving the calculated amount of zinc sulfate in distilled water. Isotonic solutions of zinc sulfate were prepared by adding the calculated amount of sodium chloride to make the solution isotonic with a 0.9 per cent sodium chloride solution. Slightly hypertonic solutions were prepared by adding sufficient sodium chloride to make the solution isotonic with a 1.4 per cent sodium chloride solution.

Calculation of tonicity was done by the osmotic factor method (2).

Zinc sulfate collyria combined with epinephrine were prepared by utilizing the proportion of epinephrine solution 1:1000 commonly used in prescriptions for the treatment of inflamed eyes:

Zinc sulfate	1.1 grains
Epinephrine solution 1:1000	20.0 minims
Sodium chloride	3.9 grains
Distilled water, *q.s.*	1.0 fluid ounce

The various buffered, stabilized collyria used were those formulated by Husa and Dale (1) and recalculated to be isotonic with a 0.9 per cent sodium chloride solution.

Formula 1.—Buffered zinc sulfate collyria stabilized and made isotonic with sodium citrate:

Molarity in $ZnSO_4$	0.009 *M*	0.026 *M*	0.032 *M*	0.035 *M*
Barbital	0.26%	0.26%	0.26%	0.26%
Sodium barbital	0.12%	0.12%	0.12%	0.12%
Sodium citrate	1.97%	1.71%	1.62%	1.59%
Zinc sulfate	0.26%	0.75%	0.92%	1.00%
Distilled water, *q.s.*	100.00 cc.			

Formula 2.—Buffered zinc sulfate collyria stabilized with sodium citrate and made isotonic with sodium chloride:

Molarity in $ZnSO_4$	0.009 *M*	0.026 *M*	0.032 *M*	0.035 *M*
Barbital	0.15%	0.15%	0.15%	0.15%
Sodium barbital	0.25%	0.25%	0.25%	0.25%
Sodium citrate	0.50%	0.50%	0.50%	0.50%
Sodium chloride	0.56%	0.46%	0.42%	0.41%
Zinc sulate	0.25%	0.75%	0.92%	1.00%
Distilled water, *q.s.*	100.00 cc.			

Formula 3.—Buffered zinc sulfate collyria stabilized with 0.15% aminoacetic acid and made isotonic with sodium chloride:

Molarity in $ZnSO_4$	0.009 *M*	0.026 *M*	0.032 *M*	0.035 *M*
Aminoacetic acid	0.15%	0.15%	0.15%	0.15%
Sodium barbital	0.41%	0.41%	0.41%	0.41%
Sodium chloride	0.62%	0.52%	0.49%	0.47%
Zinc sulfate	0.26%	0.75%	0.92%	1.00%
Distilled water, *q.s.*	100.00 cc.			

* Received May 5, 1955, from the College of Pharmacy, University of Florida, Gainesville.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.
This paper is based on a dissertation presented to the Graduate Council of the University of Florida by James R. McCowan, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education. Present address: St. Louis College of Pharmacy and Allied Sciences, St. Louis 10, Missouri.

Formula 4.—Buffered zinc sulfate collyria stabilized with 0.5% aminoacetic acid and made isotonic with sodium chloride:

Molarity in $ZnSO_4$	0.009 *M*	0.026 *M*	0.032 *M*	0.035 *M*
Aminoacetic acid	0.50%	0.50%	0.50%	0.50%
Sodium barbital	0.41%	0.41%	0.41%	0.41%
Sodium chloride	0.35%	0.24%	0.21%	0.20%
Zinc sulfate	0.26%	0.75%	0.92%	1.00%
Distilled water, *q.s.*	100.00 cc.			

Method of Testing Irritation.—The eyes of adult albino rabbits of both sexes were used to evaluate the degree of irritation produced by the collyria studied in this work. The rabbits were placed in wooden stalls which held the animals firmly but allowed complete freedom of the head and neck. One eye of a rabbit was untreated for use as a control and the solution to be tested was applied to the other eye by pinching the lower eyelid together and pulling it away from the eye forming a cup into which the solution could be introduced by a medicine dropper. In lieu of using a fixed volume of solution each time, the conjunctival sac was completely filled with solution. The lower lid was then gently pulled up and over the cornea and held in this fashion for a period of ten seconds. The instillation was repeated every ten minutes for a total of five instillations.

Determinations of irritation of the eye were made immediately after the last instillation and at one hour intervals for three additional observations with a final observation being made eighteen hours after the last instillation. Irritation was graded according to the following scale which is a modification of that used by Whitehill (3):

Photophobia	1
Discharge above normal	1
Inflammation	
of nictitating membrane	1
of conjunctiva	1
Chemosis	3
Corneal damage	4

Injury to the eye was indicated by the sum of the individual effects appearing after treatment with the various solutions.

Methods of Inducing Irritation and Determining Therapeutic Effect.—*Mustard oil chemosis.*—To induce an inflammation which could serve as a basis for testing the astringent and therapeutic properties of the solutions investigated, the eyes of a rabbit were anesthetized by placing one drop of a 1% Pontocaine® hydrochloride solution (Winthrop) in each eye. After a period of twenty minutes one drop of an emulsion containing 1.0 ml. of synthetic mustard oil and 9.0 ml. of distilled water emulsified with 0.1 ml. Tween® 20 was dropped into the conjunctival sac of each eye. The animals were allowed to rest for twelve hours for all symptoms to appear and at the end of this period only those animals exhibiting apparently the same intensity of inflammation in both eyes were used in the experiments. Mustard oil chemosis has been used as a means of testing anti-inflammatory substances by Huebner and Grabe (4) and by Jahnke (5) in determining the action of zinc sulfate on the rabbit eye.

The solution to be tested was administered by completely filling the conjunctival sac of one eye and then pulling the lower lid up and over the cornea maintaining the eye surfaces in contact with the solution for thirty seconds. The other eye was untreated and used as a control. Instillations of the collyria were made three times a day until the test eye returned to a normal condition. Observations of irritation were made daily and continued until both eyes were normal.

Ultraviolet irritation.—The effectiveness of zinc sulfate collyria to reduce inflammation of the eye caused by exposure to ultraviolet rays was determined by a method similar to that used by Cogan, Kinsey, and Drinker (6).

Radiation was obtained by means of an 85-watt type H high-pressure mercury vapor lamp capable of emitting the short ultraviolet down to approximately 2000 Å. The lamp was mounted in a porcelain socket and a $6^1/_2$-inch Pyrex tube ($1^1/_2$-inch inside diameter) was used to enclose the lamp. A hole one inch in diameter was burned into the Pyrex tube in such a position that when the tube was placed over the lamp, the hole was directly adjacent to the inner arc tube mounted within the glass bulb of the lamp. Each animal was held in a wooden stall and placed so that the eye to be exposed was in a direct line with the inner arc tube of the lamp and the aperture in the glass tubing enclosing the entire lamp. The eyelids of each eye were held open and partially everted to allow the rays to reach as much of the eye and conjunctival tissue as possible. Exposure of each eye was maintained for three minutes and at a distance of 15 cm. After exposure the animals were allowed to rest for twelve hours and then examined. Those animals exhibiting the same intensity of symptoms in both eyes were used to determine the therapeutic activity of the solutions in the same manner as was done after inducing mustard oil chemosis.

DISCUSSION OF RESULTS

Irritation.—As is shown in Table I, only a slight transient irritation was produced by distilled water and by 1.4% sodium chloride solution. Instillations of 0.9% sodium chloride solution resulted in no apparent irritation. Hypotonic and isotonic solutions containing low concentrations of zinc sulfate produced only slight irritation; however as the concentration of zinc sulfate was increased, hypotonic solutions produced a more intense irritation than did the equivalent isotonic solutions. Since the isotonic and hypotonic solutions of zinc sulfate varied only slightly in *p*H values, it would appear that differences in irritation were due to tonicity rather than *p*H factors.

The effects of *p*H on the irritation produced by the buffered stabilized zinc collyria did not appear to be of significance since collyria having the same *p*H value but different stabilizing agents showed various degrees of irritation. The inducement of irritation produced by the solutions shown in Table II is probably more dependent upon the ions in the solution and the efficiency of the stabilizing agent for the formation of zinc complexes than upon the *p*H of the solutions.

Table II shows that all of the buffered, stabilized collyria containing 0.009 *M* zinc sulfate produced slight irritation with no significant difference between the various stabilizing agents.

Buffered collyria containing 0.026 *M*, 0.032 *M*, and 0.035 *M* zinc sulfate and stabilized with sodium citrate produced slightly less irritation in the rabbit eye than was produced by nonstabilized isotonic zinc sulfate solutions of the same concentration (Tables I and II). No significant difference was observed in the effect of the use of only sufficient sodium citrate for stabilization or the use of enough of the salt to adjust the tonicity as well as to stabilize the collyria.

Buffered collyria stabilized with 0.15% aminoacetic acid produced greater irritation of the eye with the higher concentrations of zinc sulfate than did the nonstabilized isotonic solutions. The use of 0.5% aminoacetic acid for stabilization of the higher concentrations of zinc sulfate resulted in a slight lessening of irritation compared to the effect of the collyria containing 0.15% of the stabilizing agent; however, irritation was not significantly less than that produced by the nonstabilized isotonic solutions.

Of the stabilized collyria studied, those containing sodium citrate were least irritating and collyria containing aminoacetic acid were most irritating. Comparison of results (Tables I and II) obtained with stabilized and unstabilized zinc collyria indicates that buffered stabilized collyria containing 0.009 *M* zinc sulfate are, in general, slightly more irritating than a nonstabilized isotonic zinc sulfate solution of the same concentration. Buffered stabilized collyria containing higher concentrations of zinc sulfate are in some instances less irritating than the unstabilized counterpart but still produce sufficient irritation to prevent greater freedom in the use of zinc sulfate in the eye.

Vasoconstriction, blanching of the tissues, or other visible signs of astringency were not observed after instillation of any of the zinc sulfate solutions used in this investigation. These results agree with the conclusions of Jahnke (5). Although instillations of 0.009 *M* isotonic zinc sulfate did not produce visible signs of irritation under the conditions of this

TABLE I.—IRRITATION PRODUCED BY UNBUFFERED, NONSTABILIZED SOLUTIONS

Solution	pH	No. Rabbits	Degree of Irritation Hr. after Last Instillation 0	1	2	3	18
Distilled water	6.3	3	1	0	0	0	0
0.9% Sodium chloride solution	5.8	3	0	0	0	0	0
1.4% Sodium chloride	5.8	3	1	0.3	0	0	0
0.005 *M* Zinc sulfate (hypotonic)	5.95	3	1	0	0	0	0
0.009 *M* Zinc sulfate (hypotonic)	5.95	3	1	0	0	0	0
0.026 *M* Zinc sulfate (hypotonic)	5.7	3	4.3	6.3	5.6	5	0
0.032 *M* Zinc sulfate (hypotonic)	5.7	3	7	6.3	6	6	2
0.009 *M* Zinc sulfate (isotonic)	5.9	3	0	0	0	0	0
0.026 *M* Zinc sulfate (isotonic)	5.5	3	2	2	2.6	2.3	0
0.032 *M* Zinc sulfate (isotonic)	5.5	3	2	1.3	1.6	1.6	0
0.035 *M* Zinc sulfate (isotonic)	5.5	2	3	3	1	0	0

TABLE II.—IRRITATION PRODUCED BY BUFFERED, STABILIZED ZINC SULFATE COLLYRIA

Stabilizer	pH	No. Rabbits	Degree of Irritation Hr. after Last Instillation 0	1	2	3	18
Sodium citrate							
0.009 *M* $ZnSO_4$	7.4	6	0.8	0	0	0	0
0.026 *M* $ZnSO_4$	7.0	6	1	0.5	0	0	0
0.032 *M* $ZnSO_4$	6.8	6	1.3	1	0	0	0
0.035 *M* $ZnSO_4$	6.6	9	1.9	0.9	0.4	0	0
Sodium citrate with sodium chloride							
0.009 *M* $ZnSO_4$	7.45	3	1.3	0.6	0	0	0
0.026 *M* $ZnSO_4$	6.7	3	1	0.6	0	0	0
0.032 *M* $ZnSO_4$	6.55	3	1.6	1.3	0.3	0	0
0.035 *M* $ZnSO_4$	6.5	3	1.6	1.3	0.6	0	0
0.15% Aminoacetic acid with sodium chloride							
0.009 *M* $ZnSO_4$	7.3	3	1	1	0.3	0	0
0.026 *M* $ZnSO_4$	6.7	3	2.6	1.6	1.3	1	0
0.032 *M* $ZnSO_4$	6.55	3	3.6	2	1	1	0
0.035 *M* $ZnSO_4$	6.5	3	3.6	2.3	1.6	1	0
0.5% Aminoacetic acid with sodium chloride							
0.009 *M* $ZnSO_4$	6.95	3	3	0.6	0.6	0.3	0
0.026 *M* $ZnSO_4$	6.15	3	0.6	2	2	2	0
0.032 *M* $ZnSO_4$	6.1	3	1	2	2	1.6	0
0.035 *M* $ZnSO_4$	6.05	3	2	1.6	2	2	0

investigation, slightly higher isotonic concentrations as well as hypotonic solutions and buffered, stabilized zinc sulfate collyria did produce at least slight irritation.

Therapeutic Effect. *Mustard Oil Chemosis.*—Table III shows that instillations of zinc sulfate solutions of various tonicity and containing no stabilizing agents did not reduce the time required for recovery of the rabbit eye from mustard oil chemosis. Neither hypotonic, isotonic, nor slightly hypertonic solutions appeared to reduce the severity of the irritation nor was any astringent action noted after instillation of these solutions. Neither epinephrine solutions nor epinephrine combined with zinc sulfate reduced the time necessary for recovery of the rabbit eye from mustard oil chemosis, although these solutions did tend to reduce temporarily the hyperemia and discharge. No appreciable difference could be determined between the effects of epinephrine or epinephrine combined with zinc sulfate indicating that the reduction of the irritation was due to the epinephrine and not the zinc sulfate.

Instillations of buffered zinc sulfate collyria stabilized with sodium citrate and with aminoacetic acid resulted in no reduction of the time necessary (Table III) for recovery of the rabbit eye from mustard oil chemosis. Zinc sulfate stabilized and made isotonic by the addition of sodium citrate reduced the severity of the inflammation to a slight degree. Zinc sulfate solution stabilized with 0.15% aminoacetic acid increased the quantity of discharge during the course of the irritation.

TABLE III.—EFFECT OF VARIOUS SOLUTIONS ON MUSTARD OIL CHEMOSIS

Solution	No. Rabbits	Maximum Days Until Recovery Conjunctival Tissues Test	Conjunctival Tissues Control	Cornea Test	Cornea Control
0.9% Sodium chloride	3	8	8	12	12
Epinephrine in isotonic sodium chloride	3	9	8	17	17
Hypotonic 0.009 *M* $ZnSO_4$	3	11	11	20[a]	20
Isotonic 0.009 *M* $ZnSO_4$	3	7	8	16	19
Hypertonic 0.009 *M* $ZnSO_4$	3	11	11	19	21
Isotonic 0.009 *M* $ZnSO_4$ combined with epinephrine	3	9	9	18	19
Buffered 0.009 *M* $ZnSO_4$ stabilized with sodium citrate	3	8	8	19	19
Buffered 0.009 *M* $ZnSO_4$ stabilized with 0.15% aminoacetic acid	3	8	7	19	19

[a] Since the test cornea of one rabbit remained cloudy for more than 46 days while the control cornea cleared after 17 days, it was considered that damage to the test cornea was permanent.

Ultraviolet Irritation.—Isotonic and slightly hypertonic solutions of 0.009 *M* zinc sulfate did not reduce the total time necessary (Table IV) for recovery of the irritated eye although both of these solutions produced a slight reduction in the degree of hyperemia present. Hypotonic 0.009 *M* zinc sulfate solution produced an increase in the amount of discharge and a slight increase in the intensity of hyperemia but did not shorten the time necessary for recovery of the irritated eye. Marked suppression of discharge was evident after the instillation of epinephrine combined with 0.009 *M* zinc sulfate.

TABLE IV.—EFFECT OF VARIOUS SOLUTIONS ON IRRITATION INDUCED BY ULTRAVIOLET RADIATION

Solution	No. Rabbits	Maximum Days Until Recovery Test	Control
0.9% Sodium chloride	3	9	11
Epinephrine in isotonic sodium chloride	3	8	8
Hypotonic 0.009 *M* $ZnSO_4$	3	12	12
Isotonic 0.009 *M* $ZnSO_4$	3	6	7
Hypertonic 0.009 *M* $ZnSO_4$	3	7	7
Isotonic 0.009 *M* $ZnSO_4$ combined with epinephrine	3	8	8
Buffered 0.009 *M* $ZnSO_4$ stabilized with sodium citrate	3	7	7
Buffered 0.009 *M* $ZnSO_4$ with 0.15% aminoacetic acid	3	8	8

Instillations of a barbital-sodium barbital buffered 0.009 *M* zinc sulfate collyrium stabilized and made isotonic by the addition of sodium citrate resulted in an increase in inflammation in the irritated rabbit eye. Although Table IV indicates that instillations of this collyrium did not significantly affect the recovery time of the test eye, the effects produced during the course of the treatment indicated that a sodium citrate stabilized collyria exerted a deleterious effect upon the irritated eye.

A buffered collyrium stabilized with 0.15% aminoacetic acid has no effect on the recovery time of the irritated eye and did not produce results different from that of normal recovery of the control eye.

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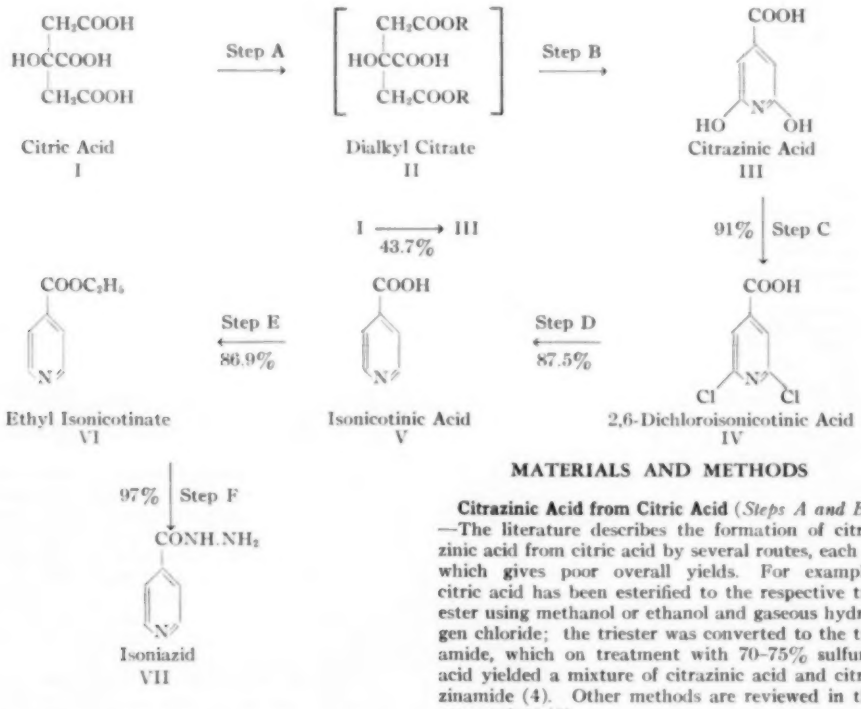
Synthesis of Isoniazid from Citric Acid*

By MANUEL M. BAIZER, MICHAEL DUB, SIDNEY GISTER, and NATHAN G. STEINBERG

A process was developed for the preparation of isoniazid from citric acid via citrazinic acid, 2,6-dichloroisonicotinic acid, isonicotinic acid, and ethyl isonicotinate. Yields and procedures in all steps were improved.

THE EMERGENCE in the last few years of isoniazid (1) as a valuable adjunct in the treatment of tuberculosis has stimulated the development of a number of new processes for the preparation of this compound (2).

The principal practical problem has been the preparation of the essential intermediate isonicotinic acid. Synthesis of this acid starting with pyridine (via 4-ethylpyridine), 4-picoline, and 2,4-lutidine, suffered from dependence upon the relatively expensive and not too freely available pure pyridine bases (3). The objective of our work, completed several years ago, was to develop a route to isonicotinic acid (and therefore to isoniazid) from readily available inexpensive starting materials. The reaction sequence finally adopted is illustrated in the following chart:



MATERIALS AND METHODS

Citrazinic Acid from Citric Acid (*Steps A and B*). —The literature describes the formation of citrazinic acid from citric acid by several routes, each of which gives poor overall yields. For example, citric acid has been esterified to the respective triester using methanol or ethanol and gaseous hydrogen chloride; the triester was converted to the triamide, which on treatment with 70–75% sulfuric acid yielded a mixture of citrazinic acid and citrazinamide (4). Other methods are reviewed in the patent cited (5).

We found (5) that citric acid in the presence of a catalytic amount of *p*-toluenesulfonic acid or an alkanesulfonic acid could be esterified to the 1,3-diester, which need not be isolated. The reaction mixture after esterification was treated directly with aqueous ammonia at 120–130° in a closed system and thereafter at atmospheric pressure with caustic soda. Acidification yielded 43.7% of crude citrazinic acid based on the total citric acid input. The crude citrazinic acid was satisfactory for use in the next step.[1]

* Received March 24, 1956, from the Research Laboratory, New York Quinine and Chemical Works Inc., Newark, N. J. The suggestions and criticisms of Dr. W. G. Bywater, S. B. Penick and Co. are gratefully acknowledged.

[1] A patent (U. S. 2,729,647, Jan. 3, 1956, A. Bavley and E. K. Hamilton to Pfizer and Co.) on the direct conversion of citric acid to citrazinic acid appeared after this work was completed.

2,6-Dichloroisonicotinic Acid (*Step C*).—Heating crude citrazinic acid at 125 ± 5° in an autoclave with three times its weight of phosphorus oxychloride gave 92.6% of crude 2,6-dichloroisonicotinic acid. Recrystallization from dilute alcohol gave a purified acid, suitable for the next step. The recovery was almost quantitative (6).

The chlorination did not succeed merely at the reflux point of phosphorus oxychloride, nor did it progress in the presence of a high-boiling inert solvent. Higher temperatures (*ca.* 170°) did not give better results than did 125°.

Isonicotinic Acid (*Step D*).—The dechlorination of 2,6-dichloroisonicotinic acid by hydrogenation was accomplished by a modification of the method of Wibaut using Raney nickel catalyst in alkaline medium (7). The total yield of isonicotinic acid obtained was 87.5%. The first crop of product was obtained as free acid, the remainder as its copper salt. Both could be used in the next step.

Ethyl Isonicotinate (*Step E*).—Direct esterification of isonicotinic acid with ethanol and sulfuric acid gave a yield of 69.4% of distilled ester. However, recovery of the unesterified isonicotinic acid as its copper salt and re-esterification of the latter brought the total yield to 86.9%.

A recent report describes the preparation of the ester via the acid chloride in 90% yield (8).

Isoniazid (*Step F*).—Heating ethyl isonicotinate in refluxing ethanol with a 10% excess of 85% hydrazine hydrate yielded, in two crops, 97% of isoniazid.

EXPERIMENTAL

Citrazinic Acid.—A mixture of 57.6 Gm. of anhydrous citric acid, 1.5 Gm. of *p*-toluenesulfonic acid monohydrate and 75 ml. of anhydrous methanol was heated under reflux, with stirring. After about ten minutes a clear solution was obtained. Stirring was stopped, but the heating continued for about two hours and thirty minutes.[2] The warm reaction mixture was transferred to an autoclave, 300 ml. of concentrated ammonia was added and the mixture heated at 120–130° for two hours. The excess ammonia was vented, after partial cooling, 35 Gm. of sodium hydroxide was added and the solution was concentrated to 350–400 ml. in the course of about five hours. After cooling and filtering the mixture was re-warmed to 80° and acidified to *p*H of less than 3 by the cautious addition, with stirring, of about 60 ml. of 50% sulfuric acid. The suspension of citrazinic acid was cooled in ice, the product removed by filtration, washed with ice water and dried to constant weight at 120–125°. The yield of brown product suitable for the next step was 20.4 Gm. (43.7%). A lighter colored product could be obtained by dissolving the crude in warm 75% sulfuric acid and pouring the solution into cold water or by recrystallizing it from concentrated hydrochloric acid. Recoveries in both cases were excellent.

2,6-Dichloroisonicotinic Acid.—An autoclave was charged with 150 Gm. of crude citrazinic acid and 450 Gm. of phosphorus oxychloride. The mixture was heated at 125 ± 5° internal temperature for four hours; the pressure developed was about 200 lb. After cooling to room temperature 500 ml. of ice water was added. The mixture was stirred on the steam bath for two hours and then chilled. The dark lower oily layer solidified, was removed by filtration, washed with ice water, and dried at 125°. The yield of crude dark brown product was 172 Gm. (92.6%). The crude product was dissolved in 520 ml. of ethanol and filtered; the filtrate was diluted with an equal volume of water and chilled. The precipitated purified product was removed by filtration, washed with cold 50% alcohol and dried at 125°. A second crop was removed by concentrating the mother liquor to one-half its volume. The purified material was tan, 16.91 Gm. (91%), m. p. 205–207° and was suitable for the next step. A purer product was obtained by dissolving the recrystallized product in dilute alkali, charcoaling, filtering and reprecipitating by the addition of acid to *p*H 1–2. This product, recovered in 99% yield was white to pale yellow, m. p. 209–210°.

Isonicotinic Acid.—To a solution of 20.0 Gm. of 2,6-dichloroisonicotinic acid in 200 ml. of water containing 29.5 Gm. of sodium hydroxide was added 20.0 Gm. of Raney nickel. The mixture was hydrogenated at 50–70° and 60–85 lb. pressure until uptake of hydrogen ceased (5–7 hours). The mixture was cooled to room temperature, and filtered; the catalyst was washed twice with 10-ml. portions of water which were added to the filtrate and once with alcohol which was discarded. The catalyst was stored under alcohol for further use.

The aqueous solution was acidified to *p*H 6.5–7 by the addition of 10% sulfuric acid, charcoaled, and filtered. The filtrate was further acidified to *p*H 3.5, and chilled. The precipitated isonicotinic acid (4.4–5.2 Gm., 34.4–40.5%, white to gray) was removed by filtration, washed with ice water, and dried at 100°.

The combined mother liquor and washes were brought to *p*H 6.5 and warmed to 80°. With stirring, 35 ml. of 30% copper sulfate was added. The mixture was cooled to room temperature, and allowed to stand for several hours. The copper isonicotinate after filtration, washing and drying weighed 8.08 Gm. equivalent to 6.54 Gm. of isonicotinic acid (51.1%). Both the crude acid and its copper salt could be used for the next (esterification) step.

Recrystallization of the crude isonicotinic acid gave a white product in 90% recovery; when mother liquor was re-used the recovery was 98–99%.

Ethyl Isonicotinate.—A suspension of 49.2 Gm. of isonicotinic acid in 183.5 ml. of absolute ethanol was stirred and cooled while 94.1 Gm. of concentrated sulfuric acid was slowly added. The temperature did not rise above 25°. Then the mixture was brought to reflux and kept at that temperature for four hours. After the first ten minutes the solid was in solution and stirring was unnecessary.

The mixture was cooled externally to 15°, and poured on 600 Gm. of ice. With stirring, about 129 Gm. of sodium carbonate was added carefully to *p*H 8–9; the temperature during the neutralization was maintained at 5–10°. The mixture was filtered

[2] In other experiments aliquots were periodically removed and titrated for free acid. In this reaction period *two* of the carboxyl groups had been esterified. A sample of the product was identical with the 1,3-dimethyl ester prepared according to Schroeter (9).

and the filtrate was extracted with three 100-ml. portions of ethylene dichloride. The organic solution was dried over anhydrous potassium carbonate and filtered. The solvent was removed at atmospheric pressure, the last portions at 125 mm. The product was collected at 105–106°/15 mm. (92° at 12 mm.) and weighed 41.8 Gm. (69.4%).

The alkaline aqueous liquor was acidified with sulfuric acid to *p*H 6.5. It was then warmed to 80° and 250 ml. of 30% copper sulfate was added. The *p*H was readjusted and the mixture cooled to room temperature. The crude copper isonicotinate, after filtration, washing and drying weighed 25.7 Gm. The copper salt was treated with ethanol and sulfuric acid as above. After the esterification, the copper sulfate was removed by filtration, the filtrate poured onto ice, made ammoniacal while cold, and extracted with ethylene dichloride. Distillation yielded an additional 9.5 Gm. of ethyl isonicotinate (15% on original charge). Further recovery of copper isonicotinate could be made from the new alkaline aqueous liquor.

Isoniazid.—A solution of 20.0 Gm. of ethyl isonicotinate in 50 ml. of absolute ethanol was stirred while 11.0 Gm. of 85% hydrazine hydrate was added rapidly. The solution was brought to and held at reflux for two hours. It was then cooled, with vigorous stirring, seeded if necessary, and finally chilled to 10°. The first crop of product was removed by filtration and washed with 5 ml. of cold ethanol: 15.1 Gm. (83%), white, m. p. 170.8–171.5° corr. A second crop was obtained after concentrating the mother liquor and washes: 2.4 Gm. (14%), white, m. p. 170.0–170.8° corr.

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Synthesis of Some Diacylimines*

By DONALD E. CADWALLADER, Jr.,† and JOSEPH P. LaROCCA‡

A series of diacylimine compounds was synthesized by reacting a primary amide with an appropriate acid chloride or anhydride.

A DISCUSSION of the anticonvulsant drugs has been adequately covered in a previous publication (1).

The structural similarity of the diacylimine compounds to the antiepileptic drugs containing the —CO—NH—CO— group indicated that this group was worthy of investigation. Activity, although low, has been reported for N-acetylphenylacetamide and N,N-methylacetylphenylacetamide (2). Some known substituted amides were synthesized and subjected to preliminary pharmacological screening. N-Acetylbenzamide and N-(chloroacetyl)-chloroacetamide did exhibit slight anticonvulsant activity. In view of the favorable results, a series of acyl-substituted amides was prepared for further evaluation.

Diacetamide has been prepared by eliminating a mole of ammonia from two moles of acetamide (3, 4) and by the acetylation of acetamide (5–9). A series of acyl and aroyl imines was prepared by reacting a nitrile and an organic acid in sealed tubes at high temperatures (10). A group of acyl-substituted acetanilides was prepared by reacting the mercury salt of acetanilide with an acid chloride (11). Several acyl-substituted benzamides were synthesized by acidification of the corresponding acylbenzamidoethylesters (12). Anhydrides have been reacted with aniline, substituted anilines and napthylamines to give the N-substituted diacylimine compounds (13, 14). Acetylbenzamide and dibenzamide were prepared by reacting the sodium salt of benzamide with the corresponding acid chlorides (15). Tarbouriech prepared a series of aliphatic diacylimines by heating the acid chloride and primary amide in sealed tubes: no solvent being employed (16). Some mono-, di-, and tri-halogenated diacylimines were synthesized by reacting nitriles and carboxylic acids under pressure (17). Polya and

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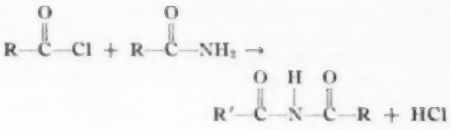
Spotswood studied the reactions between amides and anhydrides (18). Yields were increased when dry hydrogen chloride gas or an acid chloride was used as a catalyst. Further study of this reaction was carried out by Polya, *et al.* (19), and various substituted amides were prepared using an anhydride as the acetylating agent and an acid chloride as the catalyst.

EXPERIMENTAL

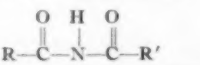
The amides, anhydrides, acids, and acid chlorides (2–4 carbon straight chain) were obtained through ordinary commercial channels. The branched chain acid chlorides were prepared by refluxing the corresponding acid with benzoyl chloride according to the procedure utilized by Philbrook (20).



N - (Diethylacetyl) - propionamide.—Two - tenths mole each of propionamide and diethylacetylchloride were refluxed six hours (a water trap was attached to the apparatus in order to carry off the hydrogen chloride gas evolved). The small amount of ammonium chloride that formed was filtered from the hot reaction mixture and the volatile portion of the reaction mixture removed until the temperature of the distillate reached 180°. The hot reaction mixture was then poured into a beaker and allowed to solidify at room temperature. After washing the solid with warm water, the impure product was placed in boiling water and ethanol slowly added until solution was complete. Recrystallization from the water-ethanol mixture yielded 3.9 Gm. (11.4%), m. p. 153–4°.

Anal. Calcd. for $C_9H_{17}O_2N$: N, 8.18. Found N, 8.20, 8.25.

TABLE I.—DIACYLIMINES



No.	R	R′	Yield, %	M. P. lit., °C.	M. P.,[a] °C.	Lit. Ref.	Nitrogen,[b] % Calcd.	Found
1	CH_3—	CH_3CH_2—	50.5	86	86	18	..	..
2	$ClCH_2$—	$ClCH_2$—	62.0	195	195	18	..	..
3	$CH_3CH_2CH_2$—	$CH_3CH_2CH_2$—	9.3	111	112	19	..	..
4	$(CH_3)_2CH$—	$CH_3CH_2CH_2$—	10.0	88	88–9	16	..	..
5	$(CH_3)_2CH$—	$(CH_3)_2CH$—	16.7	174	175	16	..	..
6	CH_3CH_2—	$(CH_3)_2CHCH_2$—	10.2	68	77–8	16	8.92	8.81 8.89
7	$(CH_3)_3C$—	CH_3CH_2—	3.0		141	..	8.92	8.95 8.80
8	$(CH_3CH_2)(CH_3)CH$—	CH_3CH_2—	11.5		114–5	..	8.92	8.90 8.85
9	$(CH_3CH_2)_2CH$—	CH_3CH_2—	11.4		153–4	..	8.18	8.20 8.25
10	$(CH_3CH_2)(CH_3)CH$—	$CH_3CH_2CH_2$—	14.0		104	..	8.18	8.21 8.22
11	$(CH_3CH_2)(CH_3)CH$—	$(CH_3)_2CH$—	8.1		139–40	..	8.18	8.15 8.31
12	$(CH_3)_3C$—	$(CH_3)_2CH$—	7.4		149	..	8.18	8.22 8.27
13	$(CH_3CH_2)_2CH$—	$(CH_3)_2CH$—	10.0		133	..	7.61	7.40 7.57
14[c]	CH_3—	CH_3—	39.0	34–6	35–7	19	..	..
15	CH_3—	C_6H_5—	40.0	117	117	18	..	..
16	$ClCH_2$—	C_6H_5—	70.0	157	157	18	..	..
17	CH_3CH_2—	C_6H_5—	32.0	98	97–8	18	..	..

[a] All melting points (uncorrected) were taken in 1-mm. capillary tubes.
[b] The nitrogen assays were determined with a Hengar-Kjeldahl apparatus using 20–30 mg. samples.
[c] Diacetanilide, the H being replaced by a C_6H_5 group.

Diacylimines.—Compounds 1, 3, 6, 15, and 17 (Table I) were prepared by refluxing 0.1 mole each of the primary amide and anhydride (slight excess) for approx. 1/2 to 1 hour. Compound 14 was prepared by refluxing acetanilide and acetic anhydride for a longer period of time. Compounds 2 and 16 were synthesized by refluxing the primary amide with chloracetylchloride, and compounds 4 and 5 were prepared by reacting the primary amide with the appropriate acid chloride.

Compounds 7–13 are reported as new. The same general procedure was used for the preparation of the new equations. The general reaction equation is:

N-Acetylisovaleramide, N-acetyl-α-phenylbutyramide and N-butyryl-α-phenylbutyramide.—All attempts to prepare these compounds by refluxing the primary amide with an anhydride resulted in liquid products. No further attempts were made to evaluate the products.

SUMMARY

1. A literature review of the preparation of diacylimine compounds has been presented.

2. Seven new diacylimine compounds were prepared.

3. All of the new compounds were recrystallized using a water-ethanol mixture and were white, light, crystalline materials.

4. All of the compounds are to be screened for anticonvulsant activity at a later date.

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The Physics of Tablet Compression XI.*

Determination of Porosity of Tablet Granulations

By W. A. STRICKLAND, Jr.,† L. W. BUSSE, and T. HIGUCHI

A method is presented for determining the porosity of tablet granulations in which two different density measurements are employed. They are (*a*) the "true" or maximum density of the granules obtained by high pressure compression of samples of the granulations, and (*b*) the apparent density, determined by mercury displacement. The values resulting from these two measurements on a given granulation were used to calculate the per cent void space in the granules. This report includes data from applying the experimental procedures to four test granulations, potassium bromide crystals (10–20 mesh), and glass beads. The results indicate that granulations prepared by the wet granulation method, using starch paste, had approximately 30% void space, while aspirin granulation, prepared by the slugging process, had approximately 3% void space.

POROSITY of tablet granulations, although given little attention to date, can be expected to influence their compressional characteristics and the physical properties of tablets prepared from them. A given weight of very porous granulation would require a deeper fill in the tablet machine than a granulation which was practically nonporous. In other words, the larger bulk of a given weight of very porous granulation necessitates a lower setting of the lower punch of the machine than is needed to achieve proper quantity of die cavity fill when using nonporous granulations. This has much consequence, particularly on single punch machines because (*a*) the compression stroke length is increased, thereby increasing the surface that must be lubricated between granules and die wall during tablet compression, (*b*) a greater amount of air must escape between punches and die wall, (*c*) the individual granules of very porous granulations are more flattened in the compression process than those granules which contain practically no pore volume, and (*d*) the lower punch must move the finished tablet a greater distance up the die during the ejection portion of the cycle, here again producing more friction than encountered with shorter ejection strokes.

Porosity is a common feature to all granulations, however, this property varies greatly in nature and degree with different methods of processing. For example, granulations prepared by the slugging process, in which materials to be granulated are compressed into large pellets under high pressures and ground to the proper size granules, contain little pore volume in the granules. On the other hand, granulations prepared by the wet mass process, in which materials to be granulated

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are usually moistened with a thick carbohydrate preparation (e. g., starch paste, acacia mucilage, syrup), then dried and ground to the proper size granules, can be expected to exhibit appreciable pore volume. It is reasonable to expect that the primary particle size of the starting materials affect the porosity of the finished granules.

The literature appears to be devoid of quantitative data concerning porosity of tablet granulations, although porosities of materials such as ceramics and catalysts have been extensively studied (1, 2), the procedures employed made no serious attempt to differentiate between inter- and intraparticular pores.

The purposes of this report are (*a*) to present a method for determining the porosity of tablet granulations, and (*b*) to give data obtained from applying this procedure to representative test granulations and other materials. The procedure appears to be suitable for determinations of intraparticular void volume of granulations used for tableting processes, of effervescent granules, of compressed or fused crystalline materials and of other substances of hard texture possessing intraparticular pores less than 50 microns in effective diameter and composed of particles considerably larger than 0.1 mm. diameter.

THEORETICAL BASIS OF PROPOSED METHOD

Porosity, which we have previously defined as the per cent void space (3), is rather an ambiguous quantity when applied to granulation granules. This is especially true when studying granulations whose size distribution includes particles which are comparable in diameter to pores found in larger particles. In a packed system it is evident that it would be extremely difficult, if not impossible, to differentiate sharply between pores formed between two or more particles and pores present in individual particles. Since the mean interparticular pore diameter is, however, directly related to the mean particle size, it is possible to select systems for which the intraparticular pores are sufficiently smaller than between particle pores to permit differential determination of the volume of the former without including a significant portion of the volume of the latter.

The proposed method is based on the fact that liquid mercury, because of its very high surface tension, will not penetrate porous substances unless under pressure. Thus mercury under a given pressure will not enter pores which are smaller than that indicated by the following approximate relationship, $D = 4t/p$, where t = surface tension of mercury (473), p = pressure (dynes/cm.^{2}), and D = diameter of pore (cm.).

For many granular systems it is possible to select pressure conditions such that mercury will fill substantially all interparticular pores and still not penetrate intraparticular openings. According to the above equation, for example, mercury under one atmosphere pressure will penetrate into all openings greater than 20 microns in diameter but not into those of smaller dimensions. Since the granule size of most tablet granulations usually exceeds 150 microns (100 mesh or larger) and the size of pore openings in the individual particles rarely exceeds 20 microns it is evident that a mercury pycnometer operating at near atmospheric pressure may prove suitable in differentiating between the two types of void spaces.

The feasibility of this approach is evident in Fig. 2 which is a plot of apparent densities of test materials and granulations *versus* the average pressure exerted by mercury on the samples. To

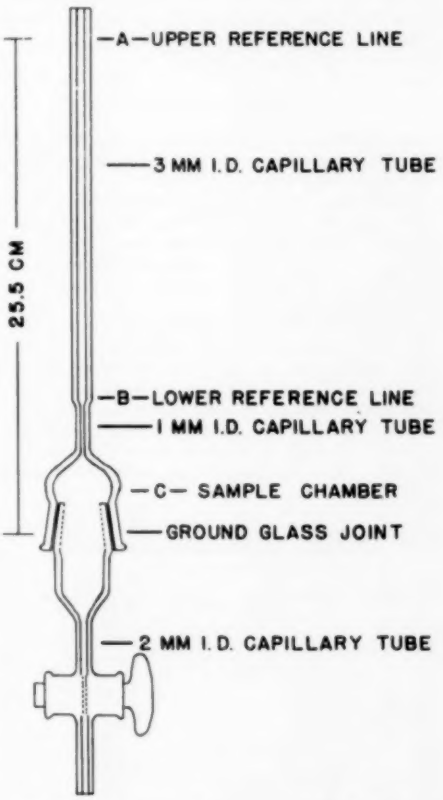

Fig. 1.—Diagram of pycnometer used to determine apparent densities of granulations. (Approx. 1/4 scale.)

pressure of *ca.* 50 cm. Hg ($1/P$ of 0.0200 cm. Hg^{-1}) the measured densities appear to show a slight linear dependency on $1/P$. At higher pressures there is a considerably greater tendency for the density values to increase. This was especially true for sodium bicarbonate granulation which appeared to have rather large pores. These results suggest that a linear extrapolation of the straight line portion, i e. the lower pressure part of the apparent density curves, to zero $1/P$ value should yield the densities of these granules including whatever pores they

contain. This is true because this portion of the apparent density curves represents the pressures and apparent densities of the granulations at which mercury is forced to surround the granules and fill only interparticular void spaces. Therefore, extrapolation to the $1/P$ axis yields an apparent density value of the granules at which essentially all intergranular spaces are filled and very little or no mercury has been forced into the pores of the granules.

In order to estimate the porosity of the sample it is necessary to know its true density in addition to the density determined by mercury displacement. Such data can be determined by helium displacement, as previously described (2, 4, 6), or approximate values may be obtained by compressing samples under sufficient pressure to eliminate void space, and then calculating density from the weight and geometric volume of the tablet.

THE METHOD

On the basis of the preceding considerations the following method was developed for determination of granulation porosity.

Apparatus.—The mercury pycnometer used in this study was made of Pyrex brand glass and utilized a 24/25 Standard taper ground glass joint as seen in Fig. 1. The height of mercury in the tube above the sample chamber was read with a cathetometer to 0.1 mm.

Procedure.—The empty pycnometer was weighed on an analytical balance to the nearest milligram. After disengaging the two parts of the ground glass joint the male portion was filled with the granulation whose apparent density was to be determined, then the outside of the joint was lightly greased with a known amount of stopcock grease (usually 20 to 30 mg.). The two parts of the joint were brought together and securely sealed by turning the two in opposite directions while pressing them together. The filled pycnometer was then weighed on an analytical balance, the difference in the two weights less the weight of the stopcock grease used corresponded to the weight of granulation in the sample chamber. Five to eight grams of granules were usually required to fill the sample chamber. The pycnometer assembly was then bound with heavy rubber bands to prevent leakage under pressure and the upper end was attached with a thick-walled rubber tube to an absolute mercury manometer reading from 0 to 100 cm. of mercury pressure. After clamping the pycnometer in an upright position the tube below the stopcock was immersed in a beaker of mercury. The manometer system and charged pycnometer were then evacuated with a Cenco Megavac vacuum pump (Central Scientific Company) until a tesla coil test revealed attainment of satisfactory vacuum. The stopcock was then cracked slightly to allow mercury to fill the pycnometer slowly and proceed up the capillary tube above the sample chamber to an arbitrary height between reference lines A and B depending on the porosity of the material being examined, at which point the stopcock was closed. While the vacuum pump was still running, the heights of reference line *A* and of the mercury column were determined with a cathetometer. Following this the vacuum pump was cut off from the system and air was allowed to enter the manometer until 10 cm. pressure was exerted on the mercury column in the capillary tube. The apparatus was gently tapped several times with a pencil to overcome sticking of mercury in the tube and another cathetometer reading made on the height of the mercury column. This procedure was repeated at intervals of 10 cm. pressure until 100 cm. was exerted on the mercury column. Pressures above atmospheric were supplied by a tank of compressed gas. After determinations were completed the pycnometer filled with granulation and mercury was weighed.

As pressure in the sample chamber increased, mercury was forced around and into the granules and the mercury column dropped in proportion to the porosity of the granulation. It was found that above 120–140 cm. of mercury total pressure the ground glass joint tended to leak mercury even though it was held together by several rubber bands. Thus, the use of the pycnometer was limited to pressures below this range.

Determination of True Density of Granules.—For the purposes of this work the true density of porous materials was determined by compressing samples of the materials between punches in a tablet die under pressures sufficient to eliminate substantially all the void space. Nelson (5) showed that the void space left in crystalline material after compression under punch forces of 7000 Kg. per sq. cm. (100,000 lb. per sq. in.) was usually less than 1%. Samples of the materials used in this study were compressed on a hydraulic press equipped with a $^3/_8$ flat faced punch and die set, at forces slightly greater than 7000 Kg. per sq. cm. The tablets obtained in this manner were weighed and their volume calculated from dimensions; the density values resulting from these procedures were considered as an approximate true density of the granules.

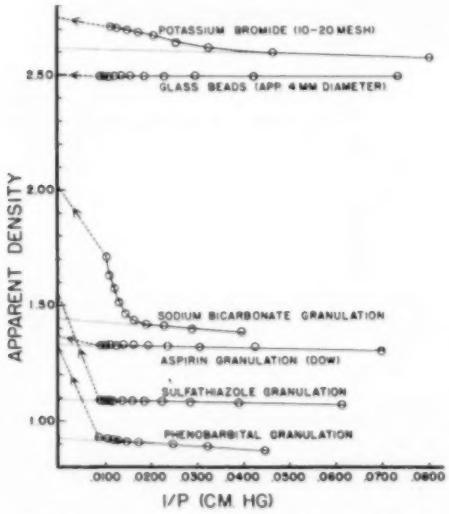

Fig. 2.—Plot of apparent densities obtained by displacement of mercury at different pressures (expressed as $1/P$). The arrow connects the apparent density at the highest pressure used with the true density of the material.

Calculations.—Calculations involved in determining density and porosity were based on the apparent displacement of mercury by the granulation at each increment of pressure. It was found convenient to calculate the porosity and apparent densities in terms of the total pressure on a median plane of the sample chamber, which was made up of the pressure on the mercury column in addition to the height of mercury above the median plane.

The first corrections applied to the cathetometer readings of the mercury column heights were derived from filling the pycnometer with mercury and then applying increasing pressures onto the mercury column; the small depression of the mercury column resulting from this blank determination was used to correct the readings taken during actual determinations.

Prior to use of the pycnometer the volume of the tube above the sample chamber and the total volume of the pycnometer were precisely determined by filling the pycnometer with mercury and weighing the filled apparatus with the mercury column at different heights in the tube. In this manner the pycnometer described here was found to have a total volume of 16.646 cc. from reference line *A* to the bottom of the stopcock and the tube above the sample chamber had a volume of 0.0545 cc. per centimeter.

The following is an outline of the calculations: $V - \Delta V = V'$, where V = total volume of the pycnometer, ΔV = volume of tube from upper reference line to top of mercury column, V' = volume of granulation and mercury at each increment of pressure; and $V' - V_{Hg} = V_s$, where V_{Hg} = volume of mercury in pycnometer, and V_s = apparent volume of sample at each increment of pressure. Therefore $wt_s/V_s = D_s$, where wt_s = weight of sample in chamber, and D_s = apparent density of sample at each increment of pressure

The porosity or interparticular void space of the granules may be calculated on a percentile basis in accordance with a previous study (3) by the following relation: per cent porosity = $[1 - (D_e/D_t)]100$, where D_e = apparent density of the granules obtained by extrapolation to infinite pressure, and D_t = true density of the granules.

The calculations for a determination using sulfathiazole granulation are shown in Table I.

Table I.—Calculations Involved in Determining Apparent Density of Sulfathiazole Granulation

Pressure Exerted on Pycnometer (cm. Hg)	Cathetometer Reading of Mercury Column Height in Pycnometer Tube (cm.)	Correction for Decrease in Blank Determination (cm.)	Corrected Mercury Column Height in Pycnometer Tube (cm.)	Difference Between Reference Line *A* and Top of Mercury Column (cm.)	$(\text{cm.})/0.0545) = \Delta V$	$V - \Delta V = V'$	$V' - V_{Hg} = V_s$	$\frac{wt_s}{V_s} = D_s$ Where D_s is the Apparent Density (Gm./cc.)	Total Pressure on Median Plane of Sample Chamber (cm. Hg)	Reciprocal of Total Pressure or $1/P$ (cm. Hg^{-1})	Diameter of Pore Mercury Will Enter at Each Increment of Total Pressure (Microns)
0	41.04	0.00	41.04	9.13	0.498	16.148	5.455	1.069	0 + 16.37 = 16.37	0.0611	87
10	40.36	0.02	40.38	9.79	0.534	16.112	5.419	1.077	10 + 15.71 = 25.71	0.0389	55
20	40.05	0.04	40.09	10.08	0.549	16.097	5.404	1.080	20 + 15.42 = 35.42	0.0282	40
30	39.88	0.06	39.94	10.23	0.558	16.088	5.395	1.081	30 + 15.27 = 45.27	0.0221	31
40	39.71	0.09	39.80	10.37	0.565	16.081	5.388	1.083	40 + 15.13 = 55.13	0.0181	26
50	39.58	0.11	39.69	10.48	0.572	16.074	5.381	1.084	50 + 15.02 = 65.02	0.0154	22
60	39.46	0.13	39.59	10.58	0.576	16.070	5.377	1.085	60 + 14.92 = 74.92	0.0133	19
70	39.36	0.15	39.51	10.66	0.581	16.065	5.372	1.086	70 + 14.84 = 84.84	0.0118	17
80	39.24	0.17	39.41	10.76	0.586	16.060	5.367	1.087	80 + 14.74 = 94.74	0.0106	15
90	39.15	0.19	39.34	10.83	0.591	16.055	5.362	1.088	90 + 14.67 = 104.67	0.0096	14
100	39.07	0.21	39.28	10.89	0.594	16.052	5.359	1.089	100 + 14.61 = 114.61	0.0087	12

Data derived from calibration of pycnometer: Total volume of pycnometer (V), 16.646 cc.; volume of tube above sample chamber, 0.545 cc./cm.; difference between reference line *A* and median plane of sample chamber, 25.50 cm.

Data derived from the procedure: Weight of granulation in pycnometer, 5.834 Gm.; weight of mercury in pycnometer, 144.771 Gm.; volume of mercury at 23° C. (V_{Hg}), 10.693 cc.; height of reference line *A*, 50.17 cm.; true density of sulfathiazole granulation obtained by compression, 1.530 Gm./cc.

RESULTS OF POROSITY DETERMINATIONS ON SOME TESTS SAMPLES AND TYPICAL GRANULATIONS

In order to test the validity and feasibility of the described method it was applied to determinations of nonporous materials and typical tablet granulations. Porosity determinations were made on the following samples: glass beads—approximately 4 mm. diameter; potassium bromide—Baker, C. P., 10–20 mesh crystals; aspirin granulation—Dow, Lot No. 3789; sulfathiazole granulation—10–20 mesh, starch paste binder; phenobarital granulation—10–20 mesh, starch paste binder; sodium bicarbonate granulation—10–20 mesh, starch paste binder.

Application of the procedure to the determination of apparent density of nonporous glass beads resulted as expected in density values substantially independent of pressure as seen in Fig. 2. The

density of these beads was found to be 2.50 Gm./cc. by water displacement while the apparent density obtained by the described procedure was 2.49 Gm./cc., thus the apparent density corresponds closely to the true density of the beads. It would appear that even the lowest pressure shown on the graph, *i. e.* 14 cm. of mercury is sufficient to envelop the beads with the pycnometer fluid.

Although potassium bromide crystals were selected for determinations on the assumption that they were also nonporous, the measured apparent density appeared to increase with increase in pressure as shown in Fig. 2. On visual inspection the crystals which had been used appeared to be a definite gray color due to mercury infiltration into the larger cracks at higher pressures. Potassium bromide crystals were shown to have a porosity of approximately 5% as seen in Table II.

TABLE II.—APPARENT DENSITY AND POROSITY OF TABLET GRANULATIONS AND OTHER MATERIALS

Granulation or Material	Apparent Density of Granules[a] (Gm./cc.)	True Density of Granules (Gm./cc.)	Per Cent Void Space in Granules[b]
Sulfathiazole granulation	1.090	1.530[c]	29
Sodium bicarbonate granulation	1.450	2.033[c]	29
Phenobarbital granulation	0.920	1.297[c]	29
Aspirin granulation (Dow)	1.330	1.370[c]	2.9
Potassium bromide (10–20 mesh)	2.615	2.75[d]	4.9
Glass beads (approx. 4 mm. diameter)	2.490	2.503[e]	0.5

[a] Apparent density of granules was determined by extrapolation of low pressure portions of apparent density versus $1/P$ curves to zero $1/P$ as seen in Figure 2.
[b] Per cent void space was calculated as shown in text.
[c] Density determined by compression of granules into tablets under high pressures.
[d] Density obtained from "Handbook of Chemistry and Physics," 34th Edition, Chemical Rubber Publishing Company.
[e] Density determined by water displacement.

The void space of starch paste granulations appeared to be on the order of 30% (Table II) and the results as shown in Fig. 2 indicate that the pore diameters are generally less than 20 microns. Sodium bicarbonate granulation exhibited an appreciable void space due to pores of 28 to 14 microns diameter as evidenced by the sharp increase in apparent density in the pressure range of 50 to 100 cm. Hg ($1/P$ of 0.0200 to 0.0100). The other granulations appeared to have no significant void space due to pores of diameter greater than 12 microns. This indicates that the pore size of the finished granules may be related to the particle size of the starting material since the sodium bicarbonate was coarser than other materials used to prepare granulations.

Visual and microscopic inspection of the granules used in these procedures revealed that mercury had been forced into the sodium bicarbonate granules at higher pressures and appeared on fractured surfaces of the granules as tiny droplets. This accounted for the distinct gray color exhibited by these granules. However, similar inspection of the other granules did not reveal noticeable mercury infiltration although tiny droplets of mercury did cling to the outer surfaces.

Aspirin granulation (Dow), prepared by the slugging process, exhibited only 2.9% void space (Table II). The great difference in porosity of granulations prepared by the two general methods is certainly to be expected.

Errors.—The accuracy of the procedure was difficult to substantiate in a quantitative manner due to the lack of a porous material of known porosity, however, the precision of results from apparent density determinations of the nonporous glass beads and the accuracy of these results when compared to density values obtained by other means indicated an accuracy within approximately ±0.5%. When the method was applied to apparent density determinations of typical tablet granulations the precision was such that results have been reproduced with a variance of apparent density averaging 0.001 Gm./cc. for all pressure increments.

Accuracy of void space data is dependent upon the accuracy with which the true density is determined. The method of determining true density used in this study may result in error on the order of 1 to 2%. Greater accuracy in determining the true density of porous materials could be achieved by helium displacement pycnometry (2, 4, 6).

CONCLUSIONS

1. A method for determining apparent density of and void space in porous granular materials has been described and employed on four representative tablet granulations.

2. The size of pores and total void space of tablet granulations show considerable variance depending on the materials used and methods of granulation.

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Evaluation of Substituted Amides Utilizing an Electronic Activity-Recording Device*

By SELDON D. FEURT and JOSEPH P. LaROCCA

A new electronic activity recording device is described for the evaluation of sedatives or analeptics. The sedative properties of five substituted amide compounds at sub-hypnotic doses has been established and compared to reference standards. Butylchloral ethyl butyramide and chloral isovaleramide possess sedative properties comparable with pentobarbital and warrant further investigation as a non-barbiturate sedative.

THE SEDATIVE and hypnotic properties of new synthetic compounds are evaluated primarily by their ability to protect an experimental animal from convulsive seizures, induced by chemical or electroshock, or to produce a degree of hypnosis that would be obvious to the investigator, as evidenced by locomotor ataxia, muscular incoordination, or profound stupor. Preferred antiepileptic agents are those protecting an ambulatory patient from convulsive seizures but exerting minimum sedative activity.

Numerous devices for recording activity of small animals have been utilized. Stewart (1) devised the revolving drum method to measure the actual number of miles run by an animal in a unit of time. Animal cages suspended by a spring and connected with writing arms to record cage movements on a revolving kymograph drum have been extensively used (2), as have square or triangular cages resting on tambours so that changes of position of the animal in the cage would be transmitted through a closed air system to activate a writing point (3). A more recent method devised at Yale (4) utilizes a small circular cage balanced on a central pivot so changes of position of the animal causes a tilting of the cage to activate one of the four microswitches arranged under the four quadrants of the cage.

The main disadvantage of these activity-recording devices for pharmacological investigation is the inability to detect minor body movements of the animal since an actual change in position of the animal from one area of the cage to another is necessary to produce measurable activity.

A new sensitive apparatus has been developed for the primary purpose of evaluating the sedative properties of new synthetic compounds of the substituted amide series after preliminary pharmacological screening for anticonvulsant properties.

EXPERIMENTAL

Recording Unit.—This activity-recording device consists of a battery of eight circular wire mesh cages 21 cm. in diameter and lined with transparent celluloid to prevent the animals from climbing the sides (Fig. 1). The circular cages are supported by

Fig. 1.—Activity cages.

Fig. 2.—Recording unit.

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Authors wish to express appreciation to Dr. W. R. Byrum, Director, Howard College of Pharmacy, Birmingham, Ala., for his assistance in designing the apparatus, and to Electric Research Corporation, Atlanta, Ga., builders of the apparatus.

TABLE I.—ANALYSIS OF VARIANCE FOR UNITS AND MICE

	S.S.		M.S.	F.
	First Hour			
Rows—cages	196,898,676	7	28,128,382	2.58
Column—mice	98,813,092	7	14,116,156	1.29
Exptl. error	534,517,842	49	10,908,527	
Total	830,229,610	63		
	Second Hour			
Rows—cages	489,644,526	7	69,949,218	2.55
Column—mice	286,091,006	7	40,870,143	1.49
Exptl. error	1,342,796,226	49	27,404,005	
Total	2,118,531,758	63		
	Third Hour			
Rows—cages	829,328,869	7	118,475,552	2.33
Column—mice	524,512,479	7	74,930,354	1.47
Exptl. error	1,269,884,013	25	50,795,360	
Total	2,623,725,361	39		
	Fourth Hour			
Rows—cages	1,218,107,035	7	174,015,291	2.22
Column—mice	901,857,720	7	128,836,817	1.64
Exptl. error	1,329,467,991	17	78,203,999	
Total	3,449,432,746	31		

modified lever type weighing scales with coil spring suspension, contact arrangements are such that each change or displacement actuates the contacts, closing an electrical circuit. The contact arrangements are adjustable to compensate for the variable mass of animals of different sizes and degree of sensitivity desired. The output or actuated contacts on the spring type scale completes a circuit to the counting unit, this counting unit is in a separate room from the activity cages to permit the recording of data without disturbing the experimental animals (Fig. 2). The counting unit consists of standard six digit electrical counters positioned in the circuit of a phase shift type of thyratron using a 2D21 vacuum tube requiring extremely low values of current to fire the thyratron. Any change in displacement caused by a motion of the animal, actuates the scale closing the contacts. The closed contacts

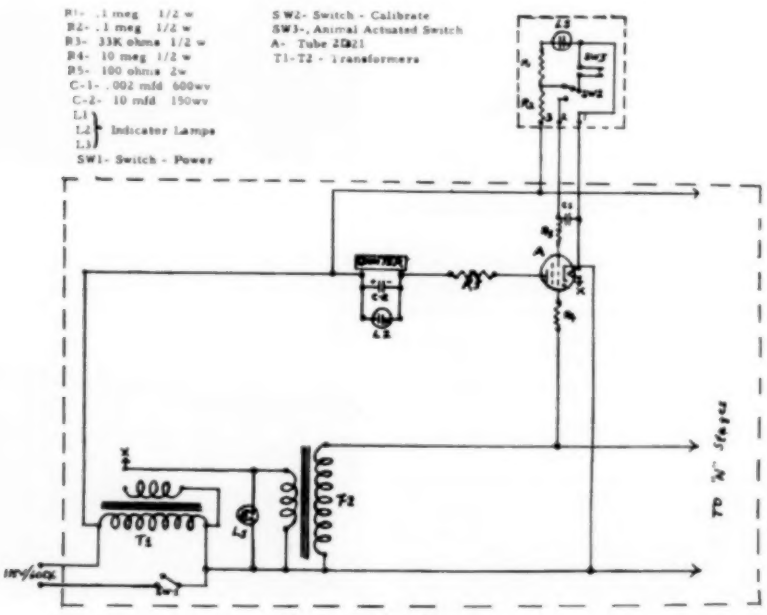

Fig. 3.—Wiring diagram.

complete a circuit to the counting unit firing the thyratron; on firing, a circuit is completed to the counters increasing the count by one, as shown in Fig. 3. The change in displacement completed, the contacts restore to normal, restoring the thyratron to a nonconductive state.

This device is capable of high counting frequencies when the scale contacts are adjusted at optimum. To facilitate this fine adjustment, a calibrating circuit consisting of a small light is connected with the contacts and permits the operator to visualize the degree of tension and sensitivity of the spring scale adjustments. Standardization of these activity units using the latin square design of Fisher (5) revealed the variability between units was greater than the variation in animals.

Normal values, standard deviations, and standard deviations of the mean, for young adult male and female Webster strain albino mice were determined for each individual recording unit. The normal values and variations at hourly intervals are listed according to sex in Table II.

Male mice were determined to be less active and more variable than females. This is verified by the previous work of Rundquist and Bellis (6). Slonaker (7) reports the presence of a female in estrus will greatly increase the activity of male mice in adjacent cages. Activity in female mice varies with stages of the estrus cycle (8) and to compensate for this variable, data are collected daily for ten consecutive days to include two full estrus cycles. All determinations are made at approximately the same time each day in a darkened, relatively soundproof room to avoid the nocturnal and diurnal variations observed by Aschoff (9).

Sterile distilled water placebos administered intraperitoneally to determine effects of injections on activity indicated only a slight evanescent stimulation, not influencing the normal activity at the end of the first hour.

TABLE II.—ANALYSIS BY SEX

Females				Males			
Cage	Mean	St. Error of Mean	% Error	Cage	Mean	St. Error of Mean	% Error
			First Hour				
1	5,717	561	9.1	1	6,434	1,544	23.9
2	3,956	483	13.3	2	2,853	268	9.3
3	4,249	487	11.4	3	5,268	758	14.3
4	4,246	836	19.6	4	2,207	418	18.9
5	3,661	421	11.5	5	1,834	385	20.9
6	4,758	556	11.7	6	3,571	709	19.8
7	7,485	1,091	14.7	7	8,636	1,444	16.7
8	6,545	573	8.8	8	5,839	595	10.1
Average of total	5,077	626	12.3		4,580	773	16.8
			Second Hour				
1	9,964	1,115	11.1	1	13,902	2,783	20.0
2	7,376	891	12.0	2	5,612	696	12.4
3	7,095	744	10.5	3	8,288	1,516	17.2
4	7,224	1,513	20.8	4	4,954	1,065	21.4
5	6,106	788	12.9	5	3,000	631	21.0
6	8,978	1,059	11.9	6	7,308	1,335	18.2
7	13,514	2,174	16.8	7	16,269	2,044	12.5
8	11,805	1,064	9.0	8	9,707	778	8.0
Average of total	9,008	1,168	12.9		8,692	1,356	15.6
			Third Hour				
1	14,821	1,548	10.3	1	17,758	3,225	18.1
2	9,793	1,147	11.8	2	7,666	891	11.6
3	9,570	1,013	10.5	3	12,048	2,172	18.0
4	9,955	1,875	18.9	4	7,729	1,875	24.2
5	8,492	1,055	12.4	5	4,323	741	17.1
6	12,938	1,432	11.0	6	9,158	1,722	18.8
7	18,464	3,078	16.7	7	21,429	2,939	13.7
8	17,390	1,470	8.4	8	14,207	1,783	12.5
Average of total	12,677	1,577	12.4		11,789	1,918	16.2
			Fourth Hour				
1	17,888	1,678	9.3	1	20,147	4,107	20.3
2	11,592	1,350	11.6	2	9,561	1,138	11.9
3	11,470	1,202	10.5	3	15,604	2,741	17.5
4	12,489	2,117	16.9	4	10,464	2,848	27.2
5	11,031	1,331	12.0	5	6,565	829	12.6
6	15,648	1,601	10.3	6	12,112	2,504	20.6
7	22,306	3,928	17.6	7	27,292	4,141	15.1
8	20,784	1,769	8.5	8	19,641	3,432	17.4
Average of total	15,401	1,872	12.1		15,173	2,717	17.9

TABLE III.—RESPONSE TO ONE-HALF HYPNOTIC DOSE I. P. INJECTION

Drug	Dose, mg./Kg.	Averages from 80 Observations 1 Hr.	2 Hrs.	3 Hrs.	4 Hrs.
Normals		5,077	9,008	12,677	15,401
Sodium pentobarbital	20	2,340	3,622	6,165	8,723
Butylchloral acetamide	250	2,946	5,047	6,953	9,411
Butylchloral ethylbutyramide	250	2,584	3,892	5,076	6,564
Chloral butyramide	200	3,421	5,405	6,974	8,560
Chloral isovaleramide	150	3,314	4,555	5,011	6,018
Chloral *n*-valeramide	150	4,454	7,267	9,864	12,209

Sodium pentobarbital was selected as the reference of choice for the four-hour determinations due to more rapid onset of action and rapid elimination, and to avoid the accumulative action of the more conventional phenobarbital. The dosage for mild sedation was calculated to be 20 mg./Kg. of body weight based on the hypnotic dose for mice as between 35 mg. and 40 mg./Kg. of body weight. Intraperitoneal injections of pentobarbital sodium at this dosage level causes no noticeable locomotor ataxia or muscular incoordination.

TABLE IV.—SEDATION EXPRESSED AS PER CENT OF NORMAL

Drug	Dose, mg./Kg.	1 Hr.	2 Hrs.	3 Hrs.	4 Hrs.
Pentobarbital	20	54.0	59.8	51.3	43.3
Butylchloral acetamide	250	41.9	43.9	45.1	38.8
Butylchloral ethyl butyramide	250	49.1	56.7	59.9	57.4
Chloral butyramide	200	32.6	39.9	44.9	45.0
Chloral isovaleramide	150	34.7	49.4	60.4	60.9
Chloral *n*-valeramide	150	12.2	19.3	22.1	20.7

These determinations are used for comparative evaluation of sedative properties of new synthetic compounds.

Pentobarbital at one-half the hypnotic dose will induce sedation in experimental animals. It is significant that the few animals stimulated with this known depressant substance died after repeated administration of the drug. This would agree with a current theory that the occasional aberrant reaction of individuals is the cause for barbiturates (10) being the greatest offender of drugs causing accidental death.

The response to pentobarbital sodium 20 mg./Kg. by intraperitoneal injection, was calculated to produce sedation of 54.0% after the first hour, 59.8% at the second hour, 51.3% at the third hour, and 43.3% at the end of four hours (see Tables III and IV).

The analeptic property of caffeine was determined by the intraperitoneal injection of 20 mg./Kg. and responses compared to normal values. Caffeine at this dosage increases activity 29.9% after one hour, 39.9% after two hours, 33.5% after three hours, and 32.4% at the end of four hours.

Five new compounds previously reported by LaRocca and Byrum (11, 12) and screened for anticonvulsant properties by K. K. Chen of Eli Lilly were evaluated for sedative properties; these compounds were administered by intraperitoneal injection at doses of one-half the previously determined hypnotic dose and responses compared to pentobarbital sodium and controls (see Tables III and IV).

The anticonvulsant properties of these compounds were determined by the minimal seizure method of Grewal (13) with pentylenetetrazole (Metrazol®) 85 mg./Kg. albino Swiss mice by subcutaneous injection. Only butylchloral acetamide conferred protection with dosage levels of 200 mg./Kg. by intraperitoneal injection with no protection by oral administration.

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A Photometric Technique for the Quantitative Measurement of Changes in Peripheral Blood Flow*

By LAURENCE E. GALE† and PAUL M. SCOTT

A technique is described which utilizes a multiplier photometer, Model 520-M, equipped with photo-multiplier tube No. 21-C and an argon light source to measure quantitatively and record the circulatory changes in the skin and striated muscles of anesthetized and unanesthetized laboratory animals. With this technique drug-produced changes in blood flow through the peripheral vascular system may be compared with simultaneously acquired manometric recordings of carotid blood pressure. The method yields information which is subject to statistical analysis.

WHILE UNDERTAKING A STUDY of the peripheral vascular effects of several cardiac and analgetic drugs, the need arose for a technique by which changes in peripheral blood flow could be observed and measured accurately. The standard methods frequently employed include the use of the flowmeter; the plethysmographic method of Brodie; the thermostromuhr method of Rien; the measurement of the blood or perfusate issuing from the vein or veins of the part; and the visual observation associating the color of the area with an increase or decrease in peripheral blood volume. None of these methods was found to be applicable to the study because they either measured the blood flow through a single large vein or artery or allowed observation only of the gross volume change of a limb. Several of the techniques, because of the inconsistency of their application, also give readings which cannot be interpreted statistically.

After preliminary investigations which consisted of plethysmographic and light transmission studies of the peripheral vascular systems of rabbits, it was considered feasible to measure the short term opacity changes of the skin and striated muscle in terms of arbitrary units of light absorption and to relate these readings to changes in peripheral blood flow. Support was given to this reasoning by the work of Molitor and Kniazuk (1), and also by the work of Edwards and Duntley (2).

EXPERIMENTAL

Description of Apparatus.—A study of the light-measuring apparatus available on the market to find one which would be sufficiently versatile and yet possess a high sensitivity indicated that the Multiplier Photometer, Model 520-M, equipped with photo-multiplier tube No. 21-C would be applicable to the study.[1]

The Multiplier Photometer, Model 520-M, is a light-measuring instrument which uses electronic amplification with an electron multiplier tube as the light-sensitive element. The photo-multiplier tube No. 21-C has the highest sensitivity of the tubes available, its response to light extending from a wavelength of 300 to 700 mμ, with maximum sensitivity at 400 mμ. The instrument is so constructed that it is completely free of the effect of line voltage fluctuations within the limits of 95 to 130 volts, and the total amplification of the unit furnishes sensitivity which permits reliable meter readings of light values as low as 1/10,000 micro lumen.

The photo-multiplier tube is housed in a metal frame containing a 1-inch window threaded to receive a standard Eastman Kodak Retainer Ring, Series 5. This feature adds greatly to the versatility of the apparatus for it allows filters and other accessory equipment to be attached directly to the housing of the light-sensitive unit.

In order to obtain permanent, continuous recordings of the opacity changes of the skin and striated muscle, a recording device was attached to the meter network of the multiplier photometer amplifier. This apparatus was composed of the amplifier section of a Brush Universal Analyzer, Model B. L. 320, connected to an Esterline-Angus Graphic Ammeter, Model AW, using linear paper No. 4331-C.

A small microswitch was incorporated in the wiring circuit connecting the multiplier photometer with the Esterline-Angus recorder. This switch was used to short out the recorder for an instant at the beginning of each drug administration, thereby marking the point of administration on the records.

The light source used was the Argon Glow Lamp.[2] This light was chosen for three reasons: Its strong emission band between 320 and 380 mμ corresponded with the high sensitivity of the photomultiplier tube to light of approximately 400 mμ wavelength (3); there is a minimum of difference in the relative amounts of light absorbed by oxyhemoglobin and hemoglobin at wavelengths of 320 to 380 mμ (4);

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[1] Manufactured by the Photovolt Corporation, 95 Madison Avenue, New York, N. Y.

[2] General Electric Glow Lamp AR-1, with specifications as follows: 2 watt, 105 to 125 volt, clear argon bulb No. S-14, medium size base, with element type P-2.

and the lamp emits a cold light with virtually all of the heat coming from the resistor in the lamp base. This heat is easily dissipated by the lamp socket and therefore does not affect the tissue.

To assure that the light intensity would remain constant throughout the experimentation a voltage stabilizer converting a. c. to d. c. was used. This apparatus protected the light from fluctuations in voltage characteristic of a. c.

The argon lamp was adapted to the experiment by enclosing it in a specially designed housing. This housing was constructed in such a manner that the light source could be used either as a mobile unit or fixed to a specially constructed light table and clamped in position beside anesthetized animals. Figure 1 pictures the argon light as adapted for the projection of light through the skin and striated muscles of anesthetized animals.

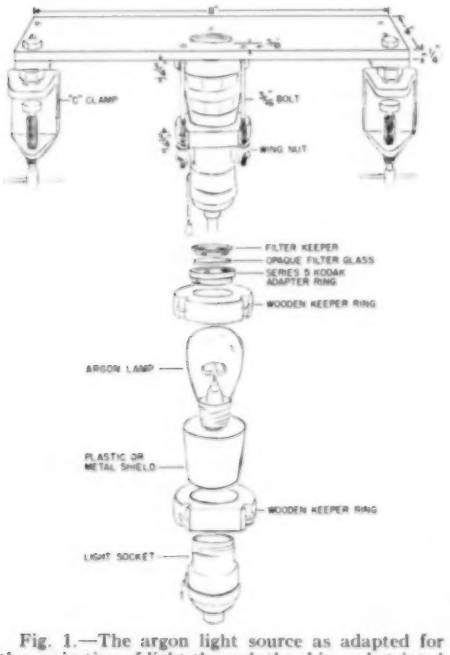

Fig. 1.—The argon light source as adapted for the projection of light through the skin and striated muscles of anesthetized animals.

A Phipps and Bird electrical kymograph with ink-writing attachment was used to record the blood pressure and respiration. To record the duration of the injections a signal magnet and timer, recording time at one second intervals, was added to the apparatus. A switch was put in the signal magnet-to-timer wiring circuit and the signal magnet was shorted out during the administration periods.

APPLICATION

Measurement of Skin Opacity and Carotid Blood Pressure in Anesthetized Animals.—To study the effects of selected drugs upon the blood flow in the skin and the general blood pressure rabbits were anesthetized with a 0.66 Gm./Kg. of urethane and 25 mg./Kg. of sodium pentobarbital. When anesthesia was deep enough to permit surgery the fur was clipped and shaved from the right hind leg, back, and throat of the rabbit. A tracheal cannula was then inserted and the animal's right carotid artery and femoral vein were isolated and prepared for cannulation. The rabbit was moved to the recording apparatus, put on its left side, and the femoral vein was cannulated into a buret system filled with 0.9 per cent saline. The rabbit was then heparinized with 5 mg. of heparin placed in solution with distilled water. A loose fold of the shaved skin was gathered on the back of the animal and lightly clipped across the light table by means of small bulldog clamps. The clamps were situated about 4 cm. from the area of the folded skin through which the light was to be projected. The multiplier photometer search unit was filtered with a 1% neutral gray filter and placed over the fold of skin on the light table.[3] Care was taken to insure that the skin was not compressed by the weight of the apparatus. The search unit and the light source were then covered with a black, rubber-impregnated canvas, and the apparatus was checked for light leakage. The carotid artery was then cannulated and attached to a mercury manometer and a double tambour arrangement was fixed to the rabbit's intercostal muscles by a single suture, so that respiration could be recorded. As soon as records of blood pressure and respiration were being obtained, the argon light was turned on and the multiplier photometer was turned to 10×, its second most sensitive setting. Adjustments were then made with the instrument's sensitivity control until the meter needle rested upon 50 arbitrary units of light transmission. This was considered to be the "0" setting for all experimentation.

If, upon the administration of a drug, blood left the area in the periphery through which light was being transmitted, the light-sensitive apparatus would acknowledge this loss by recording a decrease in the absorption of light in the area. Conversely, if, upon administration of the drug, blood entered the area, this increase in flow would be recorded as an increase in the absorption of light.

The working procedure used to obtain the recordings of the responses was as follows: (*a*) after placement of the search unit, ten minutes of recordings were taken to establish a base-line for the experiment, and the "0" was adjusted if necessary; (*b*) 1 cc./Kg. of distilled water which was used as a control was washed into the rabbit with 2 cc. of 0.9% saline; (*c*) exactly ten minutes after the administration of the control the needle of the syringe containing the dose of the drug to be studied was inserted into the translucent rubber tubing connecting the femoral cannula with the saline-filled buret; (*d*) the microswitch in the amplifier-Esterline-Angus circuit was briefly closed marking the beginning of the administration upon the Esterline-Angus recorder; (*e*) the switch shorting out the signal magnet was depressed and the injection started; (*f*) each administration was given in a period of ten

[3] Neutral gray filters removing 0.1, 1, and 10% of the projected light were purchased from the Photovolt Corporation so that the apparatus could be set at its more sensitive positions.

seconds and immediately following the emptying of the syringe, the switch in the signal magnet circuit was opened, and 2 cc. of 0.9% saline was allowed to flow into the rabbit from the buret; (g) constant recordings were then taken for ten minutes.

Measurement of Striated Muscle Opacity and Carotid Blood Pressure in Anesthetized Rabbits.—The general technique employed to obtain and record the light absorption associated with changes in the opacity of striated muscle was essentially the same as that employed in the measurement of opacity changes in the skin. As in the previous experiment, the rabbits were anesthetized with urethane and pentobarbital. When anesthesia was complete the animal's right hind leg was clipped and the throat of the animal was prepared for manometric blood pressure determination. Because the posterior portion of the animal was covered in this study all drug administrations were made into the marginal vein of the rabbit's right ear. This vein was isolated and prepared for cannulation and a disk of skin approximately 3 cm. in diameter was removed from the posterior median portion of the inside and outside of the rabbit's right thigh. The areas from which the skin was removed were immediately covered with a single thickness of surgical gauze which had been moistened in saline to prevent drying of the area. The rabbit was then placed on its left side on the surgery table with its left hind leg under the light table and its right hind leg resting upon the light source. This was accomplished in such a manner that the skinless area of the inside of the thigh completely covered the opening in the light table through which light was projected. The marginal vein in the rabbit's left ear was then cannulated into a saline-filled buret system by using a 19-gauge, metal venous cannula, and the animal was heparinized.

The light-sensitive element of the multiplier photometer was covered with a 0.1% neutral gray filter and a felt tipped, tapered extension was attached to the window in the search unit. This extension had a distal aperture of $^3/_8$ inch which was covered with a disk of opaque glass to disperse the light entering the extension. The light-sensitive unit, equipped as described above, was brought into position over the skinless area of the upper surface of the thigh, and care was taken to prevent its weight from compressing the muscles. The entire leg, light table, and search unit were then covered with the rubber-impregnated canvas and the apparatus was checked for light leakage.

From this point, the working procedure used to obtain the recordings of the responses of striated muscle differed from the procedure used to record responses in the skin only in that drug administrations were made into the buret system communicating with the marginal ear vein and that the multiplier photometer was set at 1×, its most sensitive setting.

Opacity Changes in the Ear of Unanesthetized Rabbits.—To measure the circulatory change in the ear of unaesthetized rabbits the light-sensitive unit of the multiplier photometer was covered with a 10% neutral gray filter, and a retaining ring was used to hold a felt pad, 2 cm. thick, in place around the window of the search unit. This pad was slightly concave in structure so that it would conform to the shape of the outside of the rabbit's ear.

To confine the movement of the animal a device was purchased which held the head and body of the rabbit motionless (5).[4]

The argon light in its housing was removed from the light table and fitted with accessories as portrayed in Fig. 2. The iris diaphragm was attached to the light source for this experiment to compensate for the difference in ear thickness of the rabbits. The effect of this difference in thickness could not be compensated for with the internal adjustment mechanism of the apparatus. The opaque glass disk placed between the ear and the iris diaphragm produced an illuminated area of constant size and by adjusting the diameter of the iris the intensity of the light could be varied without altering the voltage input of the bulb.

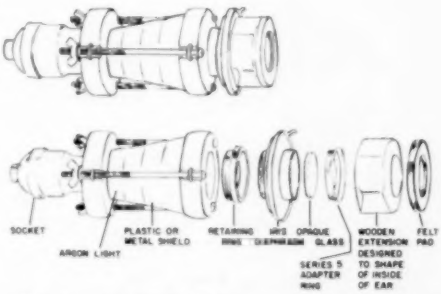

Fig. 2.—The argon light source fitted with accessories for the projection of light through the ears of unanesthetized rabbits.

To facilitate the administration of the drugs, which were injected intravenously into the marginal vein of the ear not used for opacity measurements, a syringe holder was constructed. This holder, constructed of wood, is pictured in Fig. 3.

To obtain records of drug-produced responses it was necessary, initially, to familiarize the animals with the holder. An animal was then put into the holder and the argon light and the search unit were brought into position on either side of the animal's right ear. The syringe holder was brought into position and the rabbit's left ear was fastened with scotch tape to the syringe holder above the point to be injected. Then the multiplier photometer was turned to 10×, and the apparatus checked for light leakage. Although the work was done in a darkened animal room it was impossible to prevent the entrance of outside light without compressing the ear. However, only a minimum of light leakage was tolerated.[5] The needle of the syringe, filled with the proper dosage of the drug to be administered, was then inserted into the vein of the ear, the syringe was clipped to the syringe holder, and the point of injection was covered with scotch tape.

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[4] The rabbit holder was purchased from the Kemper Manufacturing Company, Box 686, Santa Monica, Calif.

[5] The average amount of light leakage determined in 30 individual readings was 1.30 ± 0.323 arbitrary units of light transmission.

TABLE I.—AVERAGE LIGHT ABSORPTION ASSOCIATED WITH DRUG-PRODUCED OPACITY CHANGES IN THE SKIN OF RABBITS[a]

Drug	Dose mg./Kg.	No. of Measurements	Initial Transient Decrease in Absorption	Duration of Initial Transient Response in Minutes	Sustained Increase in Absorption per Admin.
Quinidine HCl	3.789	15	4.32 ± 0.517	1.38 ± 0.158	1.70 ± 0.034
	1.895	15	3.67 ± 0.340	0.97 ± 0.103	0.99 ± 0.261
Morphine SO_4	1.897	15	2.22 ± 0.751	0.72 ± 0.252	0.68 ± 0.149
	0.949	15	1.47 ± 0.433	0.60 ± 0.198	0.63 ± 0.186
Meperidine HCl	2.838	15	4.21 ± 0.323	1.27 ± 0.166	1.32 ± 0.330
	1.419	15	1.48 ± 0.245	0.34 ± 0.095	0.98 ± 0.333
Methadone HCl	0.432	15	7.09 ± 0.566	2.01 ± 0.164	1.53 ± 0.383
	0.216	15	1.97 ± 0.528	0.46 ± 0.135	1.17 ± 0.345
Papaverine HCl	3.758	15	9.08 ± 0.355	4.80 ± 0.408	0.11 ± 0.024
	1.879	15	4.38 ± 0.485	2.18 ± 0.386	0.09 ± 0.021

[a] Expressed as arbitrary units of light absorption. $t = 2.145$, $d.f. = 14$, $P = 0.05$.

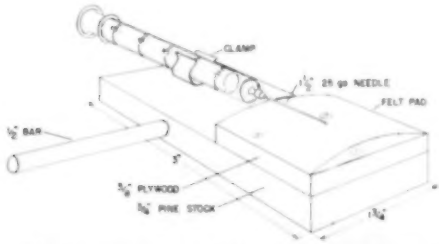

Fig. 3.—The syringe holder used to administer drugs into the marginal ear vein of unanesthetized rabbits.

The argon light and the multiplier photometer were then turned on and the Esterline-Angus recorder was adjusted. An adjustment of the iris diaphragm and the sensitivity control permitted the meter needle of the photometer to be placed, as in the other experiments, at a setting of 50 arbitrary units of transmission. The animal was then allowed to remain in this position for a period of from five to ten minutes with the recording device operating. Absolute quiet was maintained. The microswitch was then pressed to mark the point of administration and the syringe was gently depressed forcing the drug into the ear. Continuous recordings were taken for a period of twenty minutes.

Effect of the Ratio of Oxyhemoglobin to Hemoglobin in Circulating Blood.—To insure that the interpretation of the photometric peripheral vascular recordings would not be confused by the effect of a changing ratio of oxyhemoglobin to hemoglobin in the circulating blood a bypass chamber was utilized. This chamber, $1^3/_8$ inches in diameter, was constructed of glass and masonite and adapted in such a manner that by two cannulations of the same carotid artery, blood could be shunted from a rabbit into the bypass chamber and then back into the animal's circulatory system. The border of the bypass chamber and the area of the photomultiplier tube housing adjacent to the window through which the light entered were covered with a layer of $^1/_4$ inch felt padding to prevent light leakage. By use of this chamber, light was projected through a quantity of circulating arterial blood contained in an area of known depth and recorded by the multiplier photometer. The multiplier photometer was set at 1×, and no light leakage was tolerated. The animals were anesthetized, prepared for surgery, and the procedure used to record the drug-produced responses was maintained identical to that previously used.

In 40 separate administrations of respiratory depressant drugs such as morphine, meperidine, and methadone the total average deviation about the base line as measured per minute for fifty minutes ranged from a $+1.18 \pm 0.129$ to a -1.23 ± 0.124

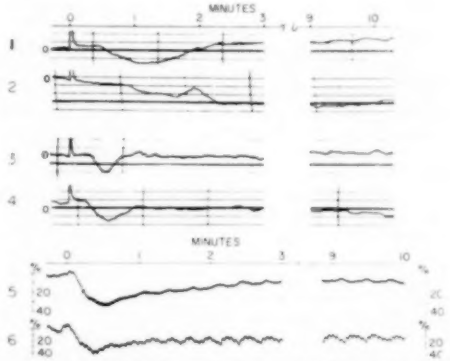

Fig. 4.—Typical recordings of the fluctuations of skin opacity, striated muscle opacity, and carotid blood pressure following the administration of 3.789 mg./Kg. of quinidine HCl and 2.838 mg./Kg. of meperidine HCl. 1 = Skin opacity following quinidine; 2 = striated muscle opacity following quinidine; 3 = skin opacity following meperidine; 4 = striated muscle opacity following meperidine; 5 = carotid blood pressure following quinidine; 6 = carotid blood pressure following meperidine. 0 = level of opacity prior to injection. The distance between each horizontal line equals two units of light absorption. A drop of the recording line below 0 indicates a decrease in light absorption; a rise above 0 indicates an increase in light absorption.

arbitrary units of light transmission. The dosages administered ranged from lethal and near lethal doses of 3.794 mg./Kg. and 3.46 mg./Kg. for morphine and methadone to dosages of each drug which had no measurable effect upon respiration. In view of the fact that the averages of response were obtained from the arterial system following toxic dosages and did not involve the peripheral circulation with its venous system, it was obvious that changes in the ratio of oxyhemoglobin to hemoglobin in the peripheral circulation would have little influence on the apparatus and therefore would not lead to fallacious interpretations of peripheral vascular changes.

Results and Discussion of Application.—Figure 4 demonstrates typical recordings of the fluctuations of skin opacity, striated muscle opacity, and carotid blood pressure following the administration of 3.789 mg./Kg. and 2.838 mg./Kg. of quinidine HCl and meperidine HCl, respectively.

The precision of the method is governed by those factors ordinarily applicable to animal experimentation. However, in over 600 individual recordings the method was found to be extremely sensitive, versatile, and practical. The duplication of results obtainable through the use of the multiplier photometer is indicated by the data presented in Table I.

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A Study of the Anatomical Effects Produced in the Leaves of *Datura stramonium* L. by the Action of 2,4-Dichlorophenoxyacetic Acid*

By CHARLES WASSBERG and FOREST J. GOODRICH†

The anatomical changes in the leaves of *Datura stramonium* L. following the application of 2,4-dichlorophenoxyacetic acid are described. Trichomes were found to be increased with abnormal bizarre forms occurring and the vascular tissue highly proliferated. The quantity of calcium oxalate crystals was significantly decreased in areas of the leaves showing severe injury. The relationship of these formative effects with adenine, auxin, and calcium uptake are briefly discussed.

DATURA STRAMONIUM has been subjected to considerable investigation involving the use of "precursor" substances and growth regulators (including 2,4-D) in the study of its alkaloid biosynthesis (1, 2). Youngken (3) described macroscopic changes occurring in the leaves of this species following the application of 2,4-D. In general these were typical formative effects characteristic of the action of this growth regulator.

The purpose of this investigation was to determine the effects of 2,4-D on the anatomical development of leaf tissues of *D. stramonium* and to ascertain which primordial regions were most seriously involved.

* Received February 24, 1956, from College of Pharmacy, University of Washington, Seattle.
This paper is based upon a thesis submitted to the Graduate School of the University of Washington by Charles Wassberg in partial fulfillment of the requirements for the degree of Master of Science.
† Professor of Pharmacognosy and Dean of the College of Pharmacy, University of Washington.

MATERIALS AND METHODS

Method of Cultivation and Treatment.—Three lots of *D. stramonium* seedlings with suitable controls (4 experimental plants and 2 controls per group) were cultivated under normal greenhouse conditions, plants in the first two groups in six-inch pots and plants of the third in hydroponic culture solution. Upon attaining a height of from eight to ten inches the plants received the following treatment:

In group one the stem of each of four plants was given an application of lanolin ointment containing 2,4-D at a concentration of 2.5% (3). The group of four soil-potted plants was watered with an aqueous solution of 2,4-D (the sodium salt) at a concentration of 0.5%, 100 cc. of this solution being poured onto the soil of each pot. In the third group hydroponic culture methods were employed during which the method of Youngken (3) was modified to include aeration by bubbling air through the culture solution.

Preparation of Plant Material for Study.—As injury to each plant in a group became apparent, ma-

terial was harvested from each plant, immediately fixed, dehydrated, and embedded in paraffin. Other specimens were cleared in chloral hydrate and mounted whole in glycerin-jelly for examination of the surfaces and internal tissues. Embedded material was sectioned at from 8 to 10 microns and stained differentially with safranine and fast green according to the methods of Johansen (7). Material was examined from every injured plant as well as from the controls in each group.

EXPERIMENTAL

Macroscopic Growth Effects.—These followed closely the results obtained by Youngken (3). Plants with which ointment treatment was used showed growth changes much more slowly than either of the other two groups. It was found useful to classify all injuries macroscopically according to the method of Watson (6), who described the effects of 2,4-D on red kidney beans. The effects on *D. stramonium* were therefore as follows:

Injury B_2 showed a roughened area along the margin of the blade near the base. Vascular tissue and trichomes in these areas were considerably overdeveloped. A section through the midrib revealed hypertrophied vascular tissue such that the concavo-convex meristele extended into the lamina as a single file of tracheary elements. These seemed somewhat smaller in diameter than normal. Collenchyma regions still maintained a well-ordered arrangement, but parenchymatous areas were distorted and crushed by the expanding vascular crescent. Calcium oxalate crystals, although present in usual locations and of characteristic type, were reduced in amount. These crystals were lesser in amount, particularly in the injured areas. Several unusually large rosettes were seen in certain sections cut near the base of the blade. Palisade tissue was continuous throughout the injured area and contained both stubby (50 μ) and elongate (80 μ) cells, abundant with chloroplasts. Spongy parenchyma appeared normal although some "replacement" tissue had developed under large veins giving the lower surface of the leaf a bulging appearance.

The upper epidermis was very uneven. The stomata in certain areas showed a tendency to elongate longitudinally. Many epidermal cells had assumed the characteristic tetragonal shape of those cells when located over large veins. Normal ones had less sinuate walls. Stomata retained the typical "cruciferous" arrangement.

The lower epidermis responded in a similar manner to 2,4-D effects, although the trichome growth was noticeably greater in this area. Nonglandular hairs were broader (up to 82 μ at the base) and somewhat longer; glandular hairs were unaltered morphologically but increased in number.

A leaf in the injury 1 category consisted of nothing more than a greatly enlarged midrib surrounded by a narrow, dark-green, rugose band of lamina. In cross section the midrib surface was concave above and extremely convex below, while the stele extended far out into the mesophyll. Most of the tracheae were of a uniform size, ± 9 μ, compared with the occurrence of two sizes, ± 6 and ± 25 μ in controls. Some collenchyma cells were discernible though most of this tissue had been crushed by other proliferating cells. The sheathing parenchyma was pushed aside and crowded outward by the expanding vascular crescent. Replacement tissue was in evidence throughout the blade. Curiously, no calcium oxalate crystals of any kind could be found in most injury 1 sections. Normal palisade and spongy tissues existed only in isolated patches; the remainder of the space was filled with vascular and "replacement" tissue. Palisade cells were single layered, much elongated, and measured between 100 and 180 μ (50 μ in controls). Spongy cells were more compact (Fig. 1).

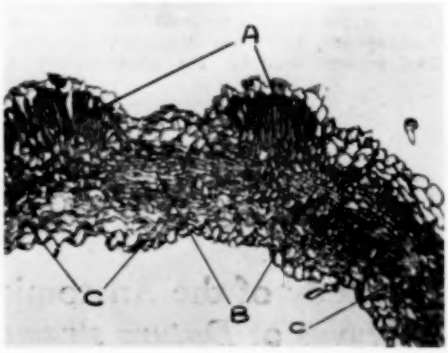

Fig. 1.—Portion of a leaf blade (injury 1). *A*, isolated patches of palisade cells; *B*, proliferated vascular tissue; *C*, "replacement" tissue. Note the absence of calcium oxalate crystals. ×65.

Leaves in the injury 2 category consisted of a narrow blade with prominent midrib, abnormally patterned veination, and a smooth surface which had slightly ruffled margins. In cross section the midrib was normal except for a uniform size of tracheae (*cf.* injury 1) and a reduced amount of crystal deposits of calcium oxalate. Spongy cells seemed less compact than those in the injury 1 category. Veins were large containing many more conducting elements than normal. The blade itself was much

Fig. 2.—Branched nonglandular trichome (injury 2). ×65.

thicker, up to 240 μ in height (controls, 145 to 150 μ). In surface view epidermal cells seemed slightly enlarged but normal in shape. The upper surface was smooth; the lower slightly uneven due to "replacement" tissue under the veins. Several extremely large and long nonglandular trichomes were observed which showed branching in a pattern characteristic for *D. stramonium* var. *inermis* Safford (8) (Fig. 2).

Mention should also be made of the occurrence of spineless fruits on all plants treated. If fertilization of the flower had occurred before treatment and the fruit therefore had commenced to develop, spines were present but markedly arrested in their development. If, however, fertilization occurred after treatment, developing fruits showed no evidence of spininess (Fig. 3).

Fig. 3.—Upper right, smooth fruit formed after treatment. Compare with normal fruit formed prior to treatment.

DISCUSSION

The derivatives of the active meristems located terminally and laterally along the axis of the primordial leaf blade failed to develop. This explains the weirdly contorted forms assumed by injured leaves. The increased pubescence shows evidence that pubescence is the result of hypertrophy of vascular tissue or is a product formed concomitantly with it. The influence of 2,4-D on the leaf hair and vascular development under the conditions of the experiment strengthens the supposition that the chemical might have a selective effect on embryonic tissues which are common for both structures and that such tissues are closely related.

The curious reduction in amount or complete absence of intracellular calcium oxalate in injured areas may have some bearing on the role of oxalic acid in plant metabolism. If oxalate is a nonessential metabolite (two carbon fragments cast aside in the initial step of the Krebs Cycle) as some have proposed, the accelerated metabolic reactions provoked by 2,4-D should result in the production of either normal or additional amounts of this product. Such was not the case. If, on the other hand, the amount of precipitated oxalate is limited by the amount of calcium available, the deficiency would also show in other ways, hence the marked distortions of cell walls in proliferated tissues which could be due to faulty development of the middle lamella of which calcium is an essential ingredient.

SUMMARY

Certain anatomical changes which occurred in the leaves of *Datura stramonium* L. following the application of 2,4-dichlorophenoxyacetic acid have been described. Significant findings included a marked decrease in the amount of intracellular calcium oxalate, proliferation of vascular tissue, and a noteworthy increase in trichome production. Briefly discussed were the relationships of these findings with each other and their importance in regard to the disturbed metabolism and altered calcium uptake provoked by 2,4-D.

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A Study of the Antibacterial Activity of Some Complex Aluminum Salts*

By THELMA N. AGUILAR, SEYMOUR M. BLAUG,† and LOUIS C. ZOPF‡

Previous reports have shown that some aluminum salts are antibacterial. The deodorant activity of these salts is partly due to antibacterial action. Aluminum formo acetate and aluminum basic formate were found to possess antibacterial activity in an oil-in-water base and a water-washable base. Aluminum chlorhydroxide complex and aluminum amino acetate showed little or no zone of inhibition in the four types of bases used. *p*H and the nonionic surfactants Tween® 80 and Span® 40 evidently had little or no influence on the zones of inhibition produced by the two formic acid salts. For the greatest antibacterial activity of an aluminum salt, an oil-in-water emulsion-type base should be employed.

The recent interest in the use of complex aluminum salts in antiperspirant formulations and the relative paucity of literature dealing with the antibacterial activity of these salts has prompted this investigation.

Alum has always been regarded as a powerful astringent, and its most important use today is as a local astringent. There are a few references to its antiseptic properties. According to Miquel (1) it will inhibit the multiplication of bacteria in the proportion of 1 part in 200.

In a paper by Shelley, *et al.* (2), the antibacterial action of certain aluminum salts was reported. They concluded that the deodorant action of aluminum salts is twofold in mechanism. There is both an antibacterial effect, thus preventing odor development, and a chemical change, thus rendering the normal odoriferous products of bacterial decomposition inoffensive.

Cromwell and Leffler (3) found that aluminum and potassium sulfate in 10 per cent aqueous solution, though not germicidal, gives as good a bacterial destruction curve as a 70 per cent solution of alcohol. Meyer and Vicher (4) agree to this finding of Cromwell and Leffler and further reported after handwashing experiments that aluminum and potassium sulfate in 10 per cent aqueous solution evidently hardens the skin to such an extent that the organisms are imprisoned but not killed.

The purpose of this study was to determine whether certain of the complex aluminum salts employed in antiperspirant formulations have any antibacterial activity and to observe the effect of various ointment bases on this activity.

* Received March 19, 1956, from the College of Pharmacy, State University of Iowa, Iowa City.
Abstracted from a thesis submitted to the Graduate College of the State University of Iowa by Thelma N. Aguilar in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
† Assistant Professor, College of Pharmacy, State University of Iowa.
‡ Dean, College of Pharmacy, State University of Iowa.

EXPERIMENTAL

Materials and Methods.—Four of the complex aluminum salts currently employed in antiperspirant formulations were selected for study: (*a*) aluminum hydroxy chloride complex,[1] (*b*) aluminum formo acetate,[2] (*c*) aluminum amino acetate,[3] and (*d*) aluminum basic formate.[4]

Each of the salts above were incorporated into the following ointment bases:

(*a*) Oil-in-water base containing: stearic acid, 14.0 Gm.; white wax, 2.0 Gm.; mineral oil, 1.0 Gm.; Myrj 52, 5.0 Gm.; propylene glycol, 3.0 Gm.; water, 75.0 Gm.

(*b*) Water-in-oil base containing: white wax, 15.0 Gm.; mineral oil, 60.0 Gm.; borax, 0.7 Gm.; water, 24.3 Gm.

(*c*) Polyethylene glycol ointment U. S. P.: polyethylene glycol 4000, 50.0 Gm.; polyethylene glycol 400, 50.0 Gm.

(*d*) Aquabase ointment containing: cholesterin, 3.0 Gm.; cottonseed oil, 3.0 Gm.; white petrolatum, 94.0 Gm.

In the preparation of the oil-in-water and water-in-oil bases part of the water was replaced so that the final emulsion contained 20% of the aluminum salt.

In the preparation of the polyethylene glycol and aquabase ointments the following type formula was employed: aluminum salt, 20%; ointment base, 80%.

The ointment bases were warmed before the salts were incorporated and the resulting mixtures were stirred until congealed.

All of the aluminum salts used in this study are soluble in water with the exception of aluminum amino acetate and all are insoluble in polyethylene glycol with the exception of aluminum formo acetate.

These ointments were tested for antibacterial activity by plating against *Staphylococcus aureus* using nutrient agar[5] containing inoculated nutrient broth[6] which had been incubated at 37° for twenty-four hours. Twelve milliliters of seeded nutrient agar was used per plate. Pyrex® glass cylinders of

[1] Reheis Company, Inc. Trade name: Chlorhydrol.
[2] Carbide and Carbon Chemicals. Trade name: Niacet.
[3] Delta Chemical Works, Inc.
[4] Victor Chemical Works.
[5] Nutrient agar 1.5%, Baltimore Biological Laboratory.
[6] Nutrient broth, Bacteriology Laboratory, State University of Iowa.

8 mm. internal diameter, 10 mm. external diameter, and 10 mm. in length were warmed slightly with the aid of sterile forceps and dropped on the seeded agar surface from a height of about 1 cm., care being taken to secure a "leak proof" seal between the edge of the cylinder and the agar. For each ointment 3 petri plates were used, one of which contained the ointment base alone and acted as control. Two cylinders were placed equidistantly on each plate.

The ointments were warmed sufficiently to soften them and then charged into the glass cylinders with the aid of standard medicine droppers the tips of which had been filed off. The plates were incubated at 37° for twenty-four hours and the zones of inhibition were measured with the aid of a transparent ruler and hand lens.

Studies to determine the influence of nonionic surfactants on the antibacterial activity of the aluminum salts were made. One per cent Tween® 80[7] was incorporated in polyethylene glycol ointment. Each of the aluminum salts under study was incorporated into this base. The above was repeated using Span® 40[8] in place of Tween® 80. The antibacterial activity of the aluminum salts in these two bases was studied by plating against *Staphylococcus aureus* using the cylinder plate method employed in the foregoing experiment. Tabulation of the results appears in Table I.

of the corresponding ointment containing the aluminum salt. Sodium borate and citric acid were used to buffer the blank ointments. The bases with adjusted *p*H were tested for antibacterial activity by plating against *Staphylococcus aureus* using the cylinder plate method. The oil-in-water base adjusted to *p*H 7–7.5 showed a slight zone of inhibition while the others showed no zones. This indicates that the zones shown in Table I are not due to the *p*H values of the bases.

DISCUSSION

Results in Table I show that the two formic acid salts possess some antibacterial activity in the oil-in-water base and the polyethylene glycol base while the two other salts, aluminum chlorhydroxide complex and aluminum amino acetate showed very slight zones of inhibition in either of these bases. The four salts studied showed no activity in the water-in-oil base or in the aquabase. Earlier investigation by various authors (5–7) showing that water-soluble antiseptics diffuse best from an oil-in-water emulsion-type base, also seem applicable to these aluminum salt-containing ointments.

A study of Table I will show that the oil-in-water base gave greater zones of inhibition than the polyethylene glycol base. The authors wanted to make certain that the zones of inhibition produced were not due to the presence of the nonionic surfactant in the oil-in-water base but to the aluminum salt, so, studies to determine the effect of nonionic surfactants on the antibacterial activity of the salts were made. Results in Table I indicate that the zones obtained with aluminum formo acetate and aluminum basic formate were not due entirely to the presence of nonionic surfactants, since there was no great increase in the zones when the nonionic surfactants were incorporated. It was also found that the zones of inhibition were not due to the low *p*H values of the aluminum salt-containing bases.

TABLE I.—ZONES OF INHIBITION IN MM.[a]

Substance and Concentration	Oil-in-Water Base, mm.	Water-in-Oil Base, mm.	Aquabase, mm.	Polyethylene Glycol Ointment USP, mm.	Polyethylene Glycol with Span 40, mm.	Polyethylene Glycol with Tween 80, mm.
Control	0	0	0	0	0	0
Aluminum formo acetate, 20%	11.83	0	0	8.2	8.8	8.5
Aluminum basic formate, 20%	13.8	0	0	2.75	4.8	4.43
Aluminum amino acetate, 20%	0	0	0	0	0	0
Aluminum chlorhydroxide complex, 20%	4.18	0	0	0	0	0

[a] Average.

TABLE II.—*p*H OF BASES AND OINTMENTS WITH ZONES OF INHIBITION

Substance and Concentration	Ointment Bases: Oil-in-Water Base, *p*H 3.72	Polyethylene Glycol Ointment USP, *p*H 6.88	Polyethylene Glycol with Tween 80, *p*H 7.42	Polyethylene Glycol with Span 40, *p*H 6.82	Water-in-Oil Base, *p*H 6.82	Aquabase, *p*H 7.42
Aluminum formo acetate, 20%	*p*H 3.71	*p*H 5.5	*p*H 4.72	*p*H 4.72	...	...
Aluminum basic formate, 20%	*p*H 3.12–3.20	*p*H 5.18	*p*H 4.39	*p*H 4.37	...	...
Aluminum chlorhydroxide, 20%	*p*H 2.92	...	...	...	...	...

[7] Tween 80 (trademark of Atlas Powder Co.), polyoxyethylene sorbitan monooleate.

[8] Span 40 (trademark of Atlas Powder Co.), sorbitan monopalmitate.

Antibacterial activity of the ointments can therefore be said to be responsible to the aluminum salts incorporated in them.

Results of the study indicate that part of the deodorant action of ointments containing aluminum salts is due to the antibacterial action of some of these compounds. For the greatest antibacterial activity of an aluminum salt an oil-in-water emulsion-type base should be used.

SUMMARY

1. Four complex aluminum salts were incorporated into several type bases: An oil-in-water, water-in-oil, water-soluble, and an absorption base.

2. Aluminum basic formate and aluminum formo acetate showed greatest zones when incorporated in the oil-in-water base and the water-soluble base.

3. Aluminum chlorhydroxide complex and aluminum amino acetate showed little or no zones of inhibition in any of the ointment bases used in this study.

4. The effect of nonionic surfactants on the antibacterial action of the complex aluminum salts was studied. Nonionic surfactants did not enhance the antibacterial effect of the ointments.

5. A study was made to determine whether *p*H had an influence on the zones of inhibition obtained with several of the complex salts. Results indicate that *p*H had little or no effect in this case.

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Radioctive Iodine in Pharmaceutical Science*

By JOHN E. CHRISTIAN†

IODINE is widely distributed in nature, and even though it occurs in extremely minute amounts, it is of vital importance to the well-being of man. It is a well known fact that small amounts of iodine are required in the daily diet in order that the body may synthesize necessary iodine-containing compounds. These compounds exist in such small amounts that usual methods of study are not sensitive enough for adequate detection and measurement. Iodine also is an element making up a part of many important drugs and the study of the rates of absorption, distribution, concentration, excretion, and fundamental mode of action in man and animals is of great importance.

In recent years a new and powerful tool for the study of iodine-containing compounds, for diagnosis, and for therapy fortunately has been made available. This new tool is in the form of radioactive iodine and because of its importance, has been one of the most widely used of the over seven hundred and thirty radioactive isotopes now known. Some of the important reasons for its extensive use are: (*a*) the importance of iodine in the economy of man and animals; (*b*) the great sensitivity of detection and measurement (10^{-16} Gm. can be detected and 10^{-14} Gm. can be quantitatively analyzed) making possible the study of low concentrations of iodine in normal metabolism which has made chemical studies difficult in contrast to the ease of radio chemical procedures; (*c*) the rather ideal radioactive properties of the most used isotope of iodine—Iodine-131; (*d*) the ability to detect and measure the gamma rays from Iodine-131 at a distance making possible the study of the body utilization of iodine in living humans and animals by external measurement; and (*e*) the concentration of iodine or iodine-containing

* Received May 25, 1956, from the Bio-Nucleonics Laboratory, School of Pharmacy, Purdue University, Lafayette, Ind.

Presented to the Scientific Section of the A. PH. A., Detroit meeting, April 11, 1956, by John E. Christian, recipient of the Eighth (1955) Chilean Iodine Educational Bureau Award for outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy and medicine.

† The work resulting in the selection of this award has been shared by many Purdue University graduate students and staff and the author wishes to express appreciation to all of them. Special thanks are due Dean Glenn L. Jenkins, fellow staff members, and graduate students who have shared my enthusiasm in the application of isotope procedures to pharmaceutical research.

compounds in certain bodily tissues permitting diagnostic, therapeutic, and extensive research applications.

Of the twenty known isotopes of iodine, only one occurs in nature. This is Iodine-127, the only stable isotope of iodine, the rest being radioactive. Of the nineteen known radioactive isotopes of iodine, Iodine-131 is the most important. Iodine-130 has been used to a minor extent and Iodine-128 although not used to any extent as yet, undoubtedly will soon find application in a recent and sensitive method called "activation analysis."

If we look at the properties of the radioactive isotopes of iodine (Table I) (1), it becomes readily apparent why many of the isotopes have not assumed importance in medicine and research.

TABLE I.—THE ISOTOPES OF IODINE

Isotope Z	A	Degree of Certainty	Half-life	Type of Decay
$_{53}I^{120}$		C	30 min.	β^+
I^{121}		B	1.5 hr.	β^+
I^{122}		A	3.6 min.	β^+
I^{123}		A	13 hr.	E.C., γ
I^{124}		A	4.5 days	E.C., β^+, γ
I^{125}		A	60 days	E.C., γ
I^{126}		A	13 days	E.C., β^+, γ
I^{127}			Stable	
I^{128}		A	25 min.	β^-, β^+, E.C., γ
I^{129}		A	1.72×10^7 yr.	β^-, γ
I^{130}		A	12.6 hr.	β^-, γ
I^{131}		A	8.1 days	β^-, γ
I^{132}		B	2.4 hr.	β^-, γ
I^{133}		A	20.5 hr.	β^-, γ
I^{134}		B	52.5 min.	β^-, γ
I^{135}		A	6.6 hr.	β^-, γ
I^{136}		C	86 sec.	β^-, γ
I^{137}		A	22 sec.	β^-
I^{138}		A	5.9 sec.	β^-
I^{139}		A	2.7 sec.	β^-

A—Element and mass number certain; B—Element certain, mass number probable; C—Element certain and mass number not well established.

The half-lives of most of the isotopes are too short to be practical. Iodine-131 has a rather ideal half-life of 8.1 days, the radiations emitted are easily detected, and this isotope is the most readily available in carrier free form at the phenomenally low cost of $0.75 per millicurie. Iodine-129 with a half-life of 1.72×10^7 years could be used; however the long half-life makes the contamination problem of greater magnitude, the radiations are much more difficult to detect, and the cost is $100.00 per millicurie. Iodine-128 can be used in "activation analysis" since it is readily formed by the thermal neutron bombardment of stable Iodine-127. The ease of detection and the short half-life of twenty-five minute Iodine-128 favors its use in this instance and this application will be discussed later.

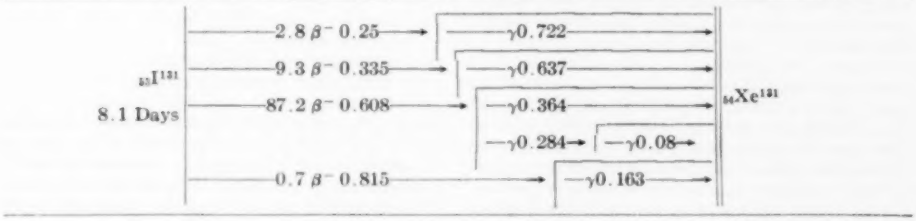
The disintegration energy and the decay scheme of Iodine-131 (Table II) (1) points up the ideal properties of this isotope.

It is not the purpose of this paper to discuss radioiodine in medical applications for diagnosis and therapy, in the accumulation of the mass of information concerning iodine-containing compounds in the animal body, nor in the study of medicinal agents containing iodine. These studies are all of high importance and point up the applications of radioiodine; however, these topics have already been thoroughly discussed by previous papers (2) and by the previous Chilean Iodine Educational Bureau Award Winners (3–6). Therefore, I propose to limit this discussion in general to the use of radioiodine as a research tool in the pharmaceutical sciences using examples of what has already been done to point up possible future applications.

Of course, the inquisitive scientist, on learning what has been done and something of the possibilities, will at once begin to ask himself how these tools may be used in his own research endeavors and thus inevitably pave the way for the development of new approaches to solving some of the problems of pharmaceutical science.

The pharmaceutical science applications as in other disciplines may be conveniently broken down into two general categories: (*a*) as tracer substances and (*b*) as analytical reagents. The differentiation between these two categories is not sharp; however, for purposes of discussion this classification is convenient.

TABLE II.—DISINTEGRATION ENERGY AND SCHEME OF IODINE-131



TRACER APPLICATIONS

Iodine-131 has been used extensively as a tracer in almost all areas of science and there are a number of examples of its use in the pharmaceutical sciences for this purpose.

One of the more recent applications is that made by Katz and Barr (7) in their studies of the rates of absorption of iodide ion from tablets prepared for sublingual absorption. Radioactive sodium iodide labeled with Iodine-131 was incorporated in one-microcurie quantities in each of twelve different tablet bases which were administered sublingually to anesthetized mice. The uptake of the iodide ion in the blood was determined by the simple expedient of placing a Geiger-Müller counter tube over the tail of the mouse and recording the relative quantities of iodide ion present in the blood with time. The tail of the animal was placed in a special lead shield which was cast to provide a shielded groove for the tail over which the detection device was placed. In this manner it was possible to show by a simple technique and with satisfactory accuracy the relative rates of sublingual absorption of iodide ion from different formulations. As the authors pointed out, determining the level of radioactivity through the tail is a much simpler and more convenient method than chemical analysis of blood samples and/or sacrificing animals and removing organs at periodic intervals. The method, of course, is much more sensitive than chemical methods and can be applied to the absorption into the circulation of other labeled materials administered parenterally, orally, rectally, and externally. The rate of disappearance from the circulation of several intravenously injected radioactive materials has also been successfully studied (8).

Other workers previously have carried out similar absorption studies using Iodine-131 by measuring the amount of this isotope in the thyroid gland. This type measurement requires sacrifice of the animal; however, greater sensitivity of detection is realized since the thyroid normally concentrates iodine up to about 10,000 times the amount found in other body organs (9) including the blood.

A rather extensive study of the relative skin absorption of iodide ion from about forty ointment bases of varying composition was made using Iodine-131 (10, 11). The technique consisted of preparing the ointment bases and incorporating 10 per cent sodium iodide labeled with Iodine-131. The radioactive preparations were applied in a definite amount and over a well defined area of a hair-free section of rat skin. After thirty hours, the animals were sacrificed, the thyroid glands removed, and the radioactivity compared with the activity of the original ointment applied to obtain the per cent of the total iodide found in the thyroid. As in virtually all applied uses of radioactive tracers, relative intensities of two or more samples are all that need be determined, making absolute determinations unnecessary. The principle requirement then is, therefore, reproducibility of sample preparation and counting conditions. The advantage of the tracer approach in this instance was the ability to detect and measure minute quantities of iodide ion penetrating the skin in the presence of iodine normally in the body and that taken in through food and water. The highest penetration in these experiments amounted to 5×10^{-8} Gm. or 5 micrograms and the lowest amount detected by this technique with satisfactory accuracy was 4×10^{-8} Gm.

Similar application of this technique was used in a study of suppository bases using radioiodine to compare the rate of absorption from rectal suppositories administered to rats (12). Five separate bases were studied by the incorporation of 3 micrograms of sodium iodide labeled with Iodine-131. At various time intervals after administration, the treated animals were sacrificed, the blood, thyroid glands, and colon containing the unabsorbed portion of the suppository were collected, and the radioactivity of aliquots of each determined. In this manner, a relative comparison of the absorption of iodide ion from the various bases was obtained.

The effect of topically applied hyaluronidase on cutaneous permeability to iodide ion has also been studied (13). The only possible approach to this type of investigation was through the use of Iodine-131 because of the minute quantities of iodine present in the 50-micron thick skin sections taken for permeability evaluation. Ointments containing Iodine-131 as iodide ion as the controls were applied to the skin of guinea pigs immediately adjacent to the test ointments identical to the controls except containing 750 turbidity reducing units of hyaluronidase. At the end of four hours the animals were sacrificed, the excess ointment carefully removed from the area, the treated skin areas removed and circular skin punches made of each. Each skin punch was frozen in a freezing microtome and 50-micron thick sections starting with the epidermis side were prepared. Approximately 50 sections of each skin punch were made and the radioactivity of each determined. In this way, the depth of permeation, the total permeability and the effect of hyaluronidase were determined in different ointment base formulations. Figure 1 gives the results of the effect of hyaluronidase on iodide skin permeability in Plastibase. This is a plot of the radioactivity in consecutive parallel sections of the control and test ointments starting with the outermost section. Hyaluronidase was found to enhance the penetration of iodide ion from this particular base. The peaks on the graph presumably represent the levels at which a comparatively large number of hair follicles are rooted.

Rather extensive studies of the iodide ion permeability of frog membrane and the effect of astringent substances on such permeability have been made using Iodine-131 as a tracer (14–17). Davson and Danielli (18) as early as 1943 had suggested that radioactive tracers should be extremely useful in such studies. Earlier studies were often unreliable, for example, electrical measurements were often subject to standard deviations of 25% or higher. The use of Iodine-131 has clearly indicated that radioactive tracer studies are more precise and rapid and permit the study of permeability in the presence of foreign substances. The experimental procedures simply involved the attachment of portions of skin taken from freshly killed frogs to the ends of 12-mm. glass tubing such that the morphological inner side contacted a solution of iodide ions labeled with Iodine-131. The external side of the skin was exposed to water or various solutions by adding these to the glass tubing. After allowing 80 minutes for equilibrium conditions to be established, 0.1-ml.

samples were taken from the inner compartment for activity measurements. In this manner it was possible to accurately calculate the permeability constant of frog skin to be 2.4×10^{-9} M. iodide/cm.^{2}/second and to determine the effect of various substances on this permeability. In addition to determining the permeability by the tracer technique, it is possible to study the route of penetration and possible sites preventing ion penetration using the autoradiographic technique. This has been done using Iodine-131 and frog skin (15). Figure 2 is an autoradiograph showing the concentration of iodine at the keratinized cells. This localization has been interpreted as indicating that either iodide ion is selectively absorbed by the keratinized cells or that the inert layer of cells acts as a barrier to the passage of iodide ion. Through a similar procedure, the investigators revealed that there is no selective avenue of penetration of frog membrane by iodide ion. Studies of this nature are not possible by other procedures.

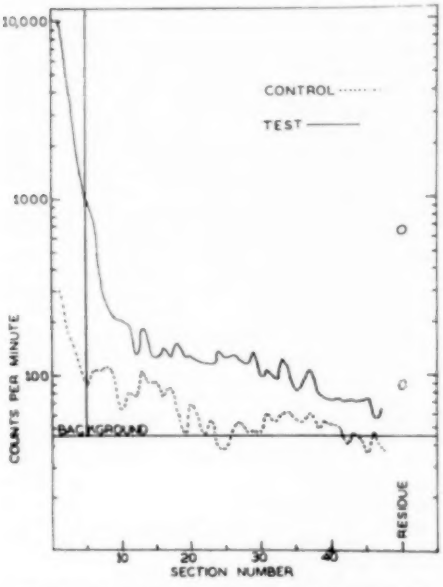


Fig. 1.—A semilog plot of radioactivity *vs.* section number. Sodium radioiodide (I^{131}) in Plastibase is the control. Test ointment is identical except containing 750 Turbidity Reducing Units of hyaluronidase.

On studying the effect of various substances on iodide ion penetration by the above procedure, it was found that chemicals having astringent properties increased the permeability, a finding which was completely unexpected since it had been generally understood that astringents have a protein precipitating action which, when applied to a membrane, lessens permeability of that membrane. Since this was an unexpected result, it was investigated in every possible manner. Without qualification, the conclusions that permeability is increased are inescapable. In addition to this, the amount of increase in permeability is proportional to the concentration of astringent making the technique applicable to comparing the effectiveness of antiperspirant compounds and formulations. More recently (17) the procedure has been applied to the evaluation of fifteen different commercially available antiperspirant creams and liquids. It is obvious that the technique may be extended to the study of the effect of a variety of pharmaceutical preparations on skin and other membranes.

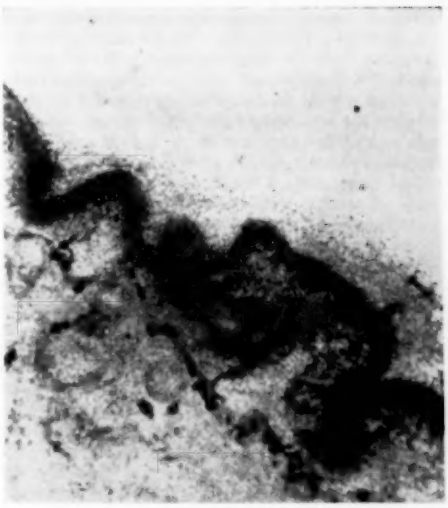


Fig. 2.—Autoradiograph of frog membrane obtained using I^{131} showing concentration of iodine at the keratinized cells. Magnification 75×. Staining was with methylene blue.

In order to develop suitable techniques for determining the retention of materials applied on the skin and also to note the effects on retention of placing polar and nonpolar soluble substances in hydrophilic and lipophilic bases, experiments on the retention of iodide ion and iodoform in o/w and w/o emulsified bases have been reported using Iodine-131 as a tracer (19). Labeled sodium iodide and iodoform were formulated in 5% concentrations in an o/w and w/o emulsified ointment base, applied to a restricted area of skin and the initial radioactivity present on the area determined with a Geiger-Müller counter. Additional activity measurements were taken at subsequent time intervals and the per cent retention calculated based on the initial activity present. Retention by this procedure was studied under normal conditions, exposure to a fine spray of water, and sensible perspiration. Here again, this approach was particularly applicable since a very sensitive and continuous method of quantitative evaluation was necessary.

Fibrinogen has been tagged with Iodine-131 (20) by a simple method. A fibrin clot formed from the tagged fibrinogen was found to constitute an adequate substrate for certain proteolytic enzymes within the range 6.3 to 8.8 *p*H. The increase in radioactivity in the filtrate of the clot after incuba-

tion with enzyme was taken as a measure of proteolytic activity. In this way it was possible to measure accurately increments of proteolytic activity equivalent to 2.5×10^{-9} to 4.7×10^{-9} hemoglobin trypsin units. The method is more sensitive and more accurate for the measurement of small amounts of plasmin and trypsin than the methods of observing the complete dissolution of a clot of fibrin as is usually used. The method is undoubtedly applicable to other similarly reacting proteolytic enzymes.

Although not strictly classified as an application in the pharmaceutical sciences, some work reported from our laboratory in 1950 and 1954 (21, 22) with Iodine-131 labeled iodocasein and sodium iodide is of interest in view of the more recent publication (23) that the milk concentration of iodine in humans is unexpectedly great after the administration of iodine to nursing mothers. This use illustrated another type of radioactive isotope application. Since iodocasein, a thyroactive iodinated protein, has been used during the past decade to increase milk and butter fat production in dairy cattle, Iodine-131 labeled iodocasein was prepared and used to study absorption, excretion, and metabolism in mammals.

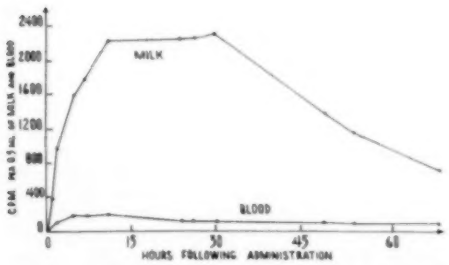


Fig. 3.—Radioactivity of skim milk and blood following the oral administration of 150 ml. of radioactive iodocasein in lactating sheep. The values represent an average of 6 ewes.

In the course of this study in lactating ewes, a radioactive assay of the milk showed unexpected high levels of iodine in comparison with the blood levels (Fig. 3). On further investigation in lactating goats, an average of 15.5% of the iodine in orally administered iodocasein was eliminated in the milk. Oral administration to lactating goats resulted in the mammary elimination of an average of 50% of administered sodium iodide. A chromatographic-autoradiographic study of the chemical form of the iodine failed to detect the presence of any iodine compounds other than iodide in the milk of goats; however, in rabbits monoiodotyrosine, diiodotyrosine, as well as iodide were identified.

ANALYTICAL APPLICATIONS

As in all areas of science, the application of radioactive isotopes as analytical tools in pharmaceutical science is perhaps the most important. This is true because analytical data is so important in research in general. With the advent of new pharmaceutical materials which are effective in small dosage forms and which are being administered in more and more complex media, many times for sustained release, there is an unusual need for the development of new methods of analyses which will enable accurate assay of micro quantities in complex mixtures. Fundamental studies of the biological distribution and of important medicinal substances for the elucidation of absorption, metabolism, and mechanism of drug action also involves the microanalysis of these materials in complex biological mixtures. The use of isotopic tracers as a tool in carrying out such analyses offers the most recent and fertile approach to solving these important analytical problems. The generalization that radioactive tracer analysis is capable of extreme sensitivity with fair to good accuracy makes the method unique and powerful. The importance of being able to analyze minute quantities in pharmaceutical science and allied science need hardly be stressed. Actually, however, the isotope approach to solving analytical problems has been rather neglected, not only by pharmaceutical science, but by other science as well, including the analytical chemical field.

Iodine-131 has thus far played a most important part in the efforts to develop new analytical methods and will undoubtedly be used to a much greater extent in the future. A brief discussion of what has been done to date with this isotope should point up the possibilities.

Measurement of Added Radioactivity.—If an element or molecule is labeled with an isotope and if the specific activity determined initially remains constant throughout the experiment, the radioactivity of any sample is directly proportional to the weight of the element or molecule. This is a very simple process in extensive utilization in radiometric analyses. Maximum sensitivity is reached when carrier-free isotope is used. With Iodine-131 as little as 10^{-14} Gm. may be quantitatively evaluated and as little as 10^{-16} Gm. may be detected by this process.

A rather unique method for the microdetermination of iodine using Iodine-131 has been reported (24). The iodine to be determined is oxidized to iodate and the iodate is allowed to react with an excess of iodide labeled with Iodine-131 to form elemental iodine. The labeled elemental iodine formed in the reaction is extracted with carbon tetrachloride and the radioactivity determined. From the amount of radioactive iodine formed the quantity of iodine present initially can be readily calculated. The essential reaction in the method is:

$$I^{127}O_3^- + 5I^{-127,131} \rightarrow 3I_2^{127,131}$$

Quantities of iodine in the order of 10^{-7} Gm. can be accurately analyzed by this method.

As little as 10 micrograms of silver can be accurately determined by precipitation with Iodine-131 labeled sodium iodide, collecting the silver iodide precipitate on ferric hydroxide and measuring the radioactivity of the precipitate (25). The reaction is as follows:

$$Ag^+ + I^{-127,131} \rightarrow AgI^{127,131} + I^{-127,131}$$

The radioactivity of the iodide ion in the filtrate may also be used to calculate the quantity of silver.

Iodine-131 has also been used to analyze for the small quantities of reduced silver in areas of different density on a photographic emulsion (26). The silver is first converted to silver bromide by treat-

ment with potassium bromide and potassium ferricyanide. The silver bromide is then converted to the iodide by treatment with radioactive iodine forming Iodine-131 labeled silver iodide. After washing the silver iodide free of excess reagents the photographic emulsion is counted. If a standard silver sample is run concurrently, the amount of silver can be directly calculated from the relative radioactivities of the sample and standard. The method is also applicable to the determination of microgram levels of silver bromide, chloride, sulfide, and other obvious silver salts.

Insulin has been reacted with Iodine-131 to form iodinated insulin which after separation and purification by the preparation of a paper electrophoretogram can be analyzed with accuracy in quantities as low as 10^{-9} Gm. (27, 28).

The measurement of protein-bound serum iodine is of considerable interest in the diagnosis of thyroid activity. A distillation step is used in the analysis and is a known source of error due to loss of iodine. To correct for this error, carrier-free Iodine-131 iodide is added just before the digestion step and is completely converted to iodate along with the organic iodide present. The percentage recovery after distillation is determined by gamma-ray measurement on the distillate and the loss of iodine due to incomplete distillation or otherwise in any step of the process is corrected in this manner (29).

In a similar type of study, Spitzy (30) used Iodine-131 to check the various stages of a method to estimate blood iodine and found that losses occurred during the distillation of HI.

A rapid method for the quantitative estimation of plasma components containing Iodine-131 is described for the analysis of human plasma. The nature of the fractions obtained is checked by chromatography and autoradiography (31).

A semimicro method has been reported for the determination of unknown equivalent weights of compounds possessing certain functional groups, e. g. alcohols, amines, and acids, by forming derivatives with *p*-iodobenzoyl chloride-Iodine-131 as the reagent (32). The derivatives of the unknowns and knowns are prepared with a single sample of the radioactive reagent. The specific activities of the unknown and the known derivatives are compared by gamma-ray counting of the crystalline compounds. The results agreed within 2% of theory.

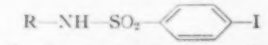
Isotope Dilution Methods.—There are a number of variations of the isotope dilution methods and these along with the advantages and necessary criteria for use have been thoroughly discussed (33, 34). The major advantages, however, are that quantitative isolation is not necessary at any step in the analysis and trace quantities (in many instances less than 10^{-9} Gm.) may be analyzed, providing for the analysis of micro and submicro levels in complex mixtures not possible by any other means. Iodine-131 has been one of the most important isotopes in the development of these methods.

Perhaps the most outstanding example is the determination of the amino acids in microgram quantities using Iodine-131 labeled *p*-iodophenyl sulfonyl chloride (pipsyl chloride) as the reagent in an inverse isotope dilution analysis procedure (35, 36).

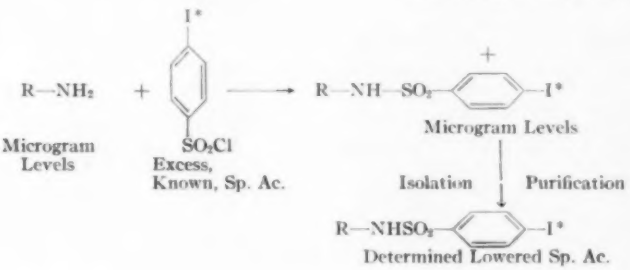
The basic equations representing the analytical procedure are as given below:

Excess labeled pipsyl chloride of known specific activity is added to a mixture of amino acids, forming the corresponding labeled derivatives. Carriers, unlabeled pipsyl derivatives corresponding to the labeled derivatives from the initial reaction, in mg. quantities are added, mixed with the labeled derivatives, separated, purified, and the specific activity determined. From the initial specific activity of pipsyl chloride, the quantity of carrier added, and the final specific activity of the isolated and purified derivative, the quantity of amino acid present can be accurately calculated. The successful analysis of several amino acids by this procedure has been reported including the determination of microgram quantities of glycine, alanine, and proline in a mixture of twelve amino acids. Such analyses are not possible by any other known procedure. The method has extremely high sensitivity, being operable below the microgram level in a complex mixture and has the inherent specificity and accuracy characteristic of isotope dilution techniques.

A number of experimental procedures based on the above method have been reported. When Iodine-131 labeled pipsyl chloride is used to form the derivatives and the corresponding derivatives labeled with Sulfur-35 are added as indicators, the method can be employed for the positive identification as well as the quantitative analysis of amino acids in protein hydrolysates (37). A scheme for the analysis of eleven monoamino acids in a single mg. sample of protein has been reported. The method has also been extended to the analysis for protein amino end groups with applications to bovine insulin, horse hemoglobin, and rabbit muscle aldose (38), to the analysis of amino acids in extremely small samples of enzymes (39), to the amino acid composition of salmine (40), and to the determination



Carrier, mg. Levels, Known Wt.



Determined Lowered Sp. Ac.

of submicrogram quantities of histamine in minute fragments of tissue (41).

Iodine-131 has been used in the development of an inverse isotope dilution analysis of amphetamine (42). Amphetamine in microgram quantities is reacted with labeled *o*-iodobenzoyl chloride to form a radioactive derivative, carrier in the form of the unlabeled *o*-iodobenzoyl amphetamine derivative is added, re-isolated, purified, and the specific activity determined. The method has a satisfactory margin of error for the determination of as low as ten micrograms of amphetamine in the pure state, in serum, and in whole blood.

Activation Analysis.—A recent and novel method of analysis which should have many applications to pharmaceutical science is called "radioactivation analysis" often referred to simply as "activation analysis" (43–45). The method involves placing the sample to be analyzed in the flux of bombarding particles for a sufficient time to produce a measurable amount of a radioisotope of the element being determined. The characterization of the radioactivity produced is a qualitative tool and the measurement of the amount of induced radioactivity is a quantitative tool. The primary advantage of activation analysis is the extreme sensitivity for many elements which exceeds that of conventional chemical and physical methods. Neutrons, being the most available type of high flux bombarding particles and giving rise to many usable nuclear reactions, are the most important in activation analysis.

The purpose for discussing this method under the use of radioactive iodine in the pharmaceutical sciences is the more or less obvious possibilities for the analysis of iodine-containing compounds by activation procedures; however, as yet this has not been reported. Since Iodine-127 is the only stable and naturally occurring isotope of iodine the atomic cross section is the same as the isotope cross section (7.0 barns) giving a distinct advantage. The reaction involved is as follows:

$$_{53}I^{127} + _{0}n^{1} \rightarrow _{53}I^{128} + \gamma$$

$T_{1/2}$ = 25 min.; β = 2.02 Mev.; γ = 0.428 Mev.

The Iodine-128 formed is easily detected and measured since a negatron with 2.02 Mev. and a gamma ray with 0.428 Mev. energies are emitted during the decay process. Since the half-life is short, saturation activity (the maximum activity obtainable with a given neutron flux) is reached in ten half-life periods or about four hours of bombardment for Iodine-128. Seven half-life periods or about three hours will give 99.2 per cent of saturation activity (43). In regard to sensitivity of the method, the cross section of the isotope, the half-life of the product, the atomic weight, the isotopic abundance and the counting efficiency all play a part, but aside from the properties of the isotope bombarded and the product produced, the most important factor is the neutron flux. Table III gives the available neutron sources, the flux, and the sensitivity values, representing the micrograms of iodine required to give an amount of radioactivity measurable by usual counting methods after exposure at saturation activity (about four hours).

The significance of these sensitivities become all the more impressive when it is realized that the lower sensitivity of the physical and chemical methods for the determination of iodine is only one microgram (46).

From Table III it is clear that in order to obtain high sensitivity, it is necessary to have an accessible nuclear reactor or accelerator and this is one big disadvantage to the method where trace quantities are to be analyzed. However, more and more reactors are becoming available and within a few years this probably will not be a serious limitation. You will note that it is already possible to carry out analyses for as little as 10-mg. quantities of iodine using laboratory sources of neutrons. The antimony-beryllium and the polonium-beryllium sources are readily available and are no more costly than many other laboratory research tools now in use; however, they need to be reactivated periodically because of the relatively short half-life of each.

Having the availability of a neutron source, the method of analysis in the usual instance is quite simple. A known quantity of the element to be analyzed is simultaneously exposed to the same flux and treated in an identical fashion as the unknown. The relative activities can then be used to calculate the unknown weight using the following equation:

$$\text{Wt. of } X \text{ in sample} = \frac{\text{Radioactivity of } X \text{ in sample}}{\text{Radioactivity of } X \text{ in std.}} \times \text{wt. of } X \text{ in std.}$$

This comparison method is more practical and more accurate than using equations derived from the growth and decay laws.

If the sample being analyzed is complex, it may be necessary, depending on the elements present, to make chemical separation to isolate the element

TABLE III.—NEUTRON SOURCES, AVAILABLE FLUX, AND SENSITIVITIES FOR THE ACTIVATION ANALYSIS OF IODINE

Neutron Sources	Flux Neutrons/ cm.²/Sec.	Sensitivity, μg. of Iodine
Materials Testing Reactor (Idaho)	2×10^{14}	0.000005 (5×10^{-12} Gm.)
Chalk River Pile (Canada)	5×10^{13}	0.00002
Oak Ridge Nat. Lab. Pile (LITR) (Oak Ridge)	1×10^{13}	0.00010
Brookhaven Nat. Lab. Pile (Brookhaven)	4×10^{12}	0.00025
Argonne Nat. Lab. Pile (Chicago)	4×10^{12}	0.00025
Harwell Pile (England)	1×10^{12}	0.001
Oak Ridge Nat. Lab. Pile (X-10) (Oak Ridge)	5×10^{11}	0.002
Cyclotron	1×10^{9}	1.0
Van de Graaff Generator	5×10^{8}	2.0
Ra-Be (1 Gm.)	1×10^{6}	10,000 (10 mg.)
Sb-Be (1 c, 60-day $T_{1/2}$)	1×10^{6}	100,000 (100 mg.)
Po-Be (1 c, 140-day $T_{1/2}$)	1×10^{6}	100,000 (100 mg.)

being determined. This can be done by the addition of a known weight of stable element, as carrier, to the standard sample and the sample to be analyzed after irradiation in order to facilitate separation and purification.

In addition to the activation analysis of the elements it is obvious that activation analysis may be applied to the study of drugs and foreign chemicals in trace amounts in biological systems in a manner similar to usual tracer methods with the advantage that the study could be carried out without subjecting the biological system to radiation effects and in addition elements, the radioactive isotopes of which are too short-lived to be used in conventional procedures, could be used. Another possibility that has not as yet been taken advantage of in the biological fields is the use of autoradiography following activation of the element involved to determine precise location and distribution.

Apart from the analysis of iodine-containing compounds, activation analysis could probably be used to much advantage as a test for the absence of trace quantities of certain toxic elements in pharmaceutical products.

The method in general appears to offer many possible applications in pharmaceutical science opening up a new avenue to the study and control of drugs, perhaps resulting in new and better pharmaceuticals.

The Standards of Iodine-131 as a Drug.—The standardization of sodium radio-iodide (I^{131}) solution as a drug official in the U. S. P. XV is of considerable interest to pharmaceutical science. There were a number of problems associated with writing the monograph for this solution and these have been discussed in a series of papers recently published (47, 48).

Special Applications in Combination with Other Methods.—It is obvious that radioiodine as an analytical tool and as a tracer has many applications in combination with other commonly used procedures of separation and analysis. Of particular significance is the use of this tool in combination with paper, ion-exchange, and adsorption chromatography. Autoradiographic techniques are extremely valuable in paper chromatographic and paper electrophoresis separation processes. These are simply examples of special applications with no attempt being made to list the multitude of applications in various analytical procedures.

DISCUSSION

Radioactive iodine, although a relatively new tool for the study of iodine and iodine-containing compounds, has already been shown to be a powerful tool in pharmaceutical science. This tool has been particularly useful in applications in which the problems could not be solved by other means. In many instances the applications are not to be considered simple in nature, since specialized instruments and skills by the research worker are required and the methods involved may be quite complex and tedious. On the other hand, the fundamental principles of isotope utilization are quite simple and as their application becomes more widespread, the procedures involved will undoubtedly compete with the more conventional methods of research and analysis. The success of isotope utilization is determined by the type of application and thoroughness of operation and like all other methods cannot be used indiscriminately for solving a variety of research problems. On the other hand, the value of the method where utilization is possible and justified cannot be denied. Radioactive isotopes in general and radioactive iodine in particular are already established as new and powerful tools which will grow in scope of application, providing another method for solving the numerous problems in pharmaceutical science.

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Notes

A Note on the Melting Range of Succinylcholine Chloride*

By JAMES E. MURPHY and C. W. FERRY

In the U.S.P. XV, the monograph for succinylcholine chloride states the following—"Succinylcholine chloride without previous drying melts between 160° and 164°." Difficulty in obtaining this range has been expressed by some workers.

Because of this, a more intensive examination was made of the various batches of Anectine® brand succinylcholine chloride that were in the laboratory. Due to the presence of two moles of water of hydration in the official substance, it was at first thought that the difficulty was caused by variations in the water content. However, pure samples of succinylcholine chloride were found to invariably contain 8.5–9.5% water (theory = 9.1% H_2O) and were stable in this range. No difficulty has been found in obtaining melting ranges within 159.5–164° on these samples. While the range of melting was usually 2–3°, the beginning and end of melting were sharp and except for gas bubbles, which disappeared about one degree above the end of melting, a perfectly clear melt was obtained.

Examination of several low melting samples from various commercial sources was made to see if a reason could be found for the low melting ranges.

In the case of a sample giving a very low melting range (153–156°) it was found that in addition to containing 11.3% water, it also contained free acid amounting to 4.6% hydrolyzed succinylcholine chloride. Other samples giving melting ranges both within and without the limits were checked for free acid as follows: 0.1 Gm. of succinylcholine chloride was transferred to a 50-ml. Erlenmeyer flask, dissolved in 5 ml. water and 1 drop of bromcresol green-methyl red mixed indicator (1) added. The solution was titrated immediately with 0.02 *N* sodium hydroxide to a gray-green color. Results are shown in Table I.

It can be seen that there is a direct relationship between the amount of free acid and the lowering of the melting range except for one sample (H). While the identity of the free acid present as an impurity was not determined, it is probably due to the presence of small amounts of free succinic acid or the mono ester of succinylcholine.

In an attempt to determine why the one sample above gave a low melting range but contained no free acid, it was decided to chromatograph several samples by the procedure used by Whittaker (2). Amounts of solution containing 1 mg. of succinylcholine chloride were spotted on Whatman No. 1 filter paper strips and developed by descending chromatography using the solvent system, *n*-propyl alcohol-benzyl alcohol-water, 5:2:2. After developing for 16–40 hours, the strips were dried in air and then sprayed heavily with a 1% solution of iodine in alcohol. Sample H above and a sample of an injection from another source gave, in addition to the succinylcholine chloride spot, an unidentified spot of moderate intensity just below and adjoining the point of application of the sample. The identity of this impurity is not known but it is suggested that it is the cause for the low melting range. None of the other samples in Table I or samples of injection from four other sources gave this extra spot. Samples of injections did give an additional spot due to the presence of some of the mono ester of succinylcholine which forms on storage.

Table I.—Melting Range and Free Acid of Succinylcholine Chloride

Sample	Melting Range, ° C.	ml. 0.02 *N* NaOH/0.1 Gm.
A	157.0–160.0	0.32
B	159.5–162.0	0.20
C	157.0–162.0	0.29
D	153.0–156.0	1.13
E	161.0–163.5	0.11
F	159.5–162.3	0.18
G	159.5–163.0	0.09
H	154.5–157.5	0.14

The above data indicate that the melting range of succinylcholine chloride gives a very good indication of the purity of the compound. Except for possibly spreading the range about one degree to make it 159–164°, it is felt that it should be kept in the monograph as a test for purity.

* Received May 25, 1956 from the Analytical Research Laboratory of Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

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A Note on the Synthesis of Pyrrole-2-carboxylic Acid*

By E. E. SMISSMAN,† M. B. GRABER, and R. J. WINZLER

IN THE DEGRADATION of sialic acid (1, 2) isolated from submaxillary mucin and other sources, one of the principal products obtained is pyrrole-2-carboxylic acid. In this laboratory it was desired to obtain this pyrrole acid for comparison with sialic acid and degradation products isolated by a different procedure. Several methods for its preparation are reported in the literature (3, 4) but either the yields were low or the reactions gave a mixture of both the 2- and 3-carboxylic acids.

In an attempt to secure a method which would afford higher yields of a pure product, high pressure carboxylation of pyrrole was tried and afforded a 53% yield of pure pyrrole-2-carboxylic acid.

EXPERIMENTAL

Six and seven-tenths grams (0.1 mole) of freshly distilled pyrrole was added to a mixture of 100 ml. water and 50 Gm. (0.5 mole) of potassium carbonate in a 300-ml. steel reaction vessel.[1] The vessel was sealed and carbon dioxide was introduced to a pressure of 900 p. s. i. The reaction vessel was shaken for five minutes at room temperature during which time an initial pressure drop of 325 p. s. i. was observed. The vessel was repeatedly recharged and shaken for five-minute intervals until a constant pressure reading of 800 p. s. i. was obtained. The temperature of the vessel was elevated to 100° and maintained at this temperature while shaking the vessel for three hours.

The reaction mixture was filtered and the vessel washed with 100 ml. of water and the washings passed through the filter. The combined filtrates were heated to boiling with 0.5 Gm. of charcoal, filtered, and cooled to 0–5°. The solution was acidified to *p*H 2–3 with concentrated hydrochloric acid. This process required rapid agitation and cooling. The acidified mixture was cooled to 0° and the acid and mixed salts were removed immediately by filtration. The solid material was mixed with 100 ml. water and concentrated ammonium hydroxide was added until the material had dissolved. The solution was heated to boiling and 0.25 Gm. of charcoal was added. The solution was filtered, cooled to 0–5° and acidified with concentrated hydrochloric acid to *p*H 2–3. The solution was cooled to 0° and filtered immediately to give a white, crystalline solid, m. p. 197–98° decompn. [lit. 192° decompn. (4)], yield 5.2 Gm. (53.6%). Mixed m. p. 196–197° decompn.

* Received November 17, 1955, from department of Biochemistry, College of Medicine, University of Illinois, Chicago.

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[1] American Instrument Company, Silver Spring, Maryland.

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Book Notices

Biochemistry for Medical Students. 6th ed. By W. V. THORPE. J. B. Lippincott Company, Philadelphia, 1956. viii + 542 pp. 14.5 x 21 cm. Price $6.75.

This book represents a revision of a standard textbook on biochemistry. The major additions to the 6th edition relates to protein structure, enzyme A and its role in metabolic processes. The entire chapter on enzymes has been thoroughly revised and contains much essential new information on this subject. In addition, the material on nucleic acids has been rewritten and the subject of redox potentials has been brought up to date. The bibliography is adequate for the purpose intended and includes references to recently published works.

Actualités Pharmacologiques. Published under the direction of RENÉ HAZARD. Masson et Cie., Paris, 1955. 198 pp. 16.5 x 25 cm. Price 1,480 fr.

This volume in the pharmacology series that was started in 1949 continues to present (in French) discussions on new groups of medicinals, their mode of action and theoretical and practical considerations of the pharmacodynamic problems involved. The text is composed of seven papers by different authors and are titled: adrenaline and noradrenaline synthetic tuberculostatics; antibiotics obtained from higher plants; lysis and the inversion of the tensional effects of adrenaline; pharmacodynamics of theophylline and its derivatives; comparative study

of the pharmacology of veratrum and rauwolfia; vitamin B_{12} and antipernicious principle. An alphabetical list of the authors and their papers for the series of eight volumes is appended.

Accidents Therapeutiques en Dermatologie. Published under the direction of JACQUES CHARPY. Masson et Cie., Paris, 1955. 342 pp. 16 x 24 cm. Price 2,000 fr.

This book is a report (in French) of the fifth symposium of the Marseillaise branch of the French Society of Dermatology and Syphiligraphy. The major part of the program is a series of 10 reports and 13 communications from dermatologists and clinicians discussing sensitivity and intolerance as well as toxic reactions to chemotherapeutic agents administered systemically and locally.

Methods of Biochemical Analysis. Vol. 3. Edited by DAVID GLICK. Interscience Publishers, Inc., New York. x + 437 pp. 16 x 23.75 cm. Price $9.50.

This annual series offers a selection of reports which includes developments and new ideas of current interest in methods and procedures for the determination and assay of biologically important substances and systems. The first two volumes of the series were reviewed in THIS JOURNAL, **43,** 445(1954); and **44,** 385(1955).

Volume III is divided into twelve parts under the following headings: determination of organic phosphorus compounds by phosphate analysis; assay of thioctic acid; quantitative determination of histamine; enzymic micro determinations of uric acid, hypoxanthine, xanthine, adenine, and xanthopterine by ultraviolet spectrophotometry; use of periodate oxidations in biochemical analysis; end group analysis of polysaccharides; use of infrared analysis in the determination of carbohydrate structure; measurement of complex ion stability by the use of ion exchange resins; analysis of metal-protein complexes; applications of metal buffers and metal indicators in biochemistry; determination of zinc in biological materials; and flame photometry and spectrometry. The very useful arrangement of text material in each chapter follows the plan established in the earlier volumes. References are given at the end of each chapter, and author and subject indexes for Volume III, and cumulative indexes for the three volumes are appended. The book contains much information that should be useful to analysts and investigators in pharmacy, particularly in the industrial laboratories.

Essays in Biochemistry. Edited by SAMUEL GRAFF. John Wiley & Sons, Inc., New York, 1956. x + 345 pp. 14.5 x 23.5 cm. Price $6.50.

This is a compilation of twenty-five essays on the present status of and future trends in biochemistry. The wide range of the discussions is indicated in the titles which follow: some metabolic products of basidiomycetes; heterogeneity of deoxyribonucleic acid (DNA); biosynthesis of branched-chain compounds; the biochemistry of lysogeny; the development of a plasma volume expander; the very big and the very small: remarks on conjugated proteins; unbalanced growth and death: a study in thymine metabolism; some thoughts on the biochemistry of the steroid hormones; the biochemistry of the bacterial viruses; the biosynthesis of peptide bonds; on the nature of cancer; problems in lipid metabolism; tetrazoles as carboxylic acid analogs; the structural basis for the differentiation of identical groups in asymmetric reactions; the nitrogen-sparing effect of glucose; the metabolism of inositiol in microorganisms: a study of molecular conformation and biochemical reactivity; the biochemistry of ferritin; some aspects of nitrogen transfer in biosynthetic mechanisms; on the bigness of enzymes; the biosynthesis of porphyrins; the role of carbohydrates in the biosynthesis of aromatic compounds; on determining the chemical structure of proteins; glycogen turnover; the veratrum alkamines; and the chemical basis of heredity determinants. The material is well presented, with the syle of the editor evident in the uniformly-clear manner of statement. The references at the end of each article and the general index add to the book as a reference volume. The format, type, and illustrations are very good. It is an interesting compilation of useful material.

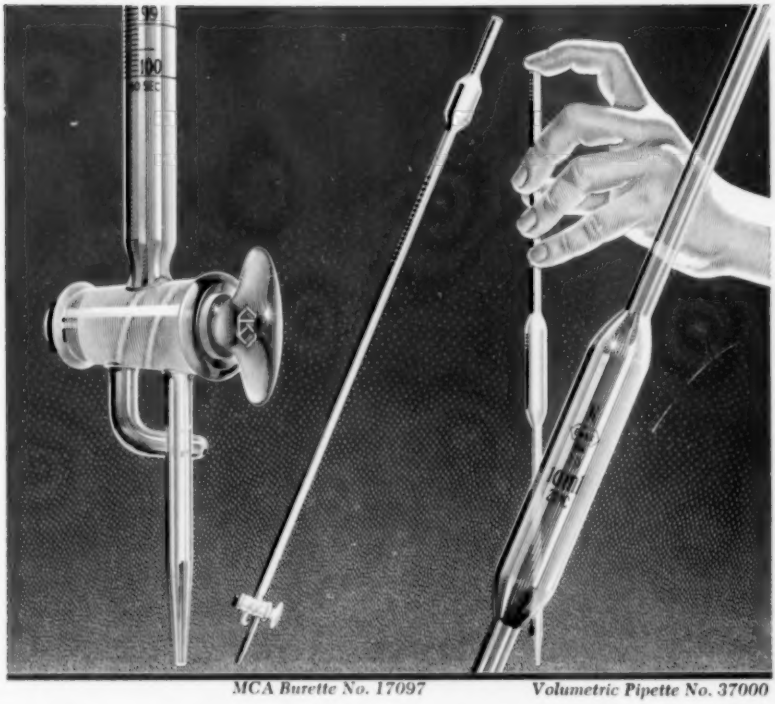
Mechanism of Organic Chemical Reactions. By E. DE BARRY BARNETT. Interscience Publishers Inc., New York, 1956. vi + 289 pp. 14 x 22.5 cm. Price $4.75.

This book is a concise presentation of modern views of the mechanism by which some familiar reactions of organic chemistry take place. It is designed for students and as a refresher for postgraduate reading. The text is written in an interesting and readable style. It is well illustrated with many structural formulas. The book should receive the attention of undergraduate teachers of organic chemistry and reference libraries.

A Textbook of Pharmacognosy. By N. M. FERGUSON. The Macmillan Company, New York, 1956. vii + 374 pp. 14 x 23.5 cm. Price $7.

This book is designed as a teaching aid, and, since the chief emphasis in the study of pharmacognosy has shifted from drug identification to learning the properties of drug constituents, the author classifies the textual matter biochemically. The introductory chapters expound "Fundamental considerations in the study of pharmacognosy" and "The biosynthesis of plant constituents." These are followed by chapters in which drugs are divided under the headings: carbohydrates, glycosides, fats and related products, volatile oils, resins and related products, alkaloids, proteins and amino acids, enzymes, blood derivatives, bacterial products, allergens, antibiotics, vitamins, hormones, and products used as toxicants.

Descriptive discussions of drugs are supplemented by helpful tabulations. The text material is followed by lists of books for further reading at the end of each chapter and a general index is appended. Teachers of pharmacognosy should be interested in this new textbook.



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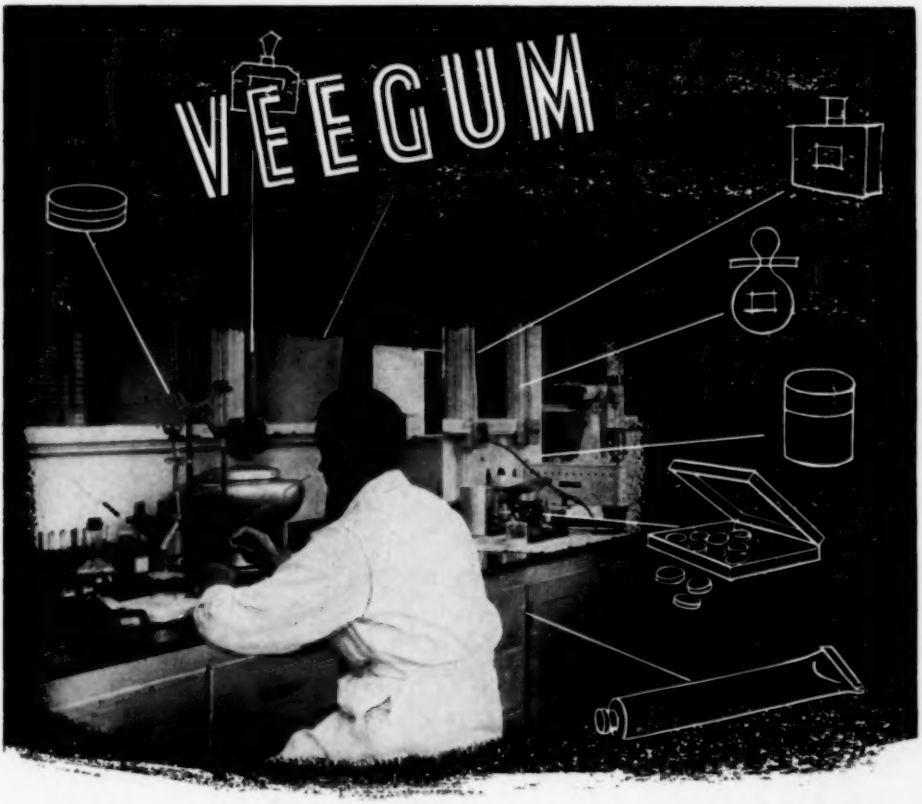

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